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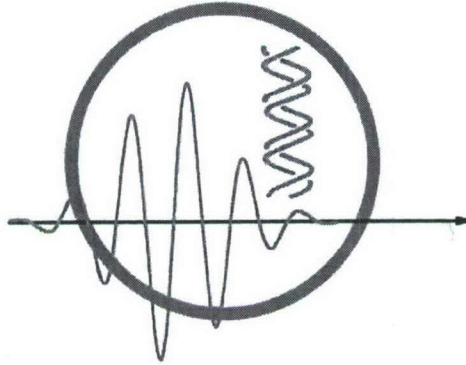
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<b>13. SUPPLEMENTARY NOTES</b>					
<b>14. ABSTRACT</b> The interest in subcellular effects of radiofrequency radiation has led to the establishment of a Multidisciplinary University Research Initiative (MURI) with scientists from six academic institutions (Old Dominion University, Eastern Virginia Medical School, Massachusetts Institute of Technology, Washington University, University of Wisconsin and University of Texas Health Science Center) participating. The ODU-led MURI worked closely with its sister MURI, led by Purdue Calumet University. The research work in the ODU - MURI has led to the establishment of a new field in the area of pulsed electric field interactions with cells and tissues - "Intracellular Electromanipulation" - with important biomedical applications. In particular, the research has a) led to a better understanding of the effect of nanosecond pulses on cellular and subcellular membranes through experimental and modeling results, b) has revealed the effect of such pulses on cell functions, such as apoptosis and calcium release from internal stores, c) has provided important information about nanosecond pulsed electric field effects on genes and proteins, d) has demonstrated the use of nanopulses for the treatment of tumors and the activation of platelets (for advanced wound healing), and e) has opened the possibility of using antennas with intense electrical pulses in the subnanosecond range, instead of electrodes, for medical treatments.					
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FY'02 MURI Consortium:

# **Subcellular Responses to Narrowband and Wideband Radiofrequency Radiation**



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submitted to

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## TABLE OF CONTENTS

OLD DOMINION UNIVERSITY CONSORTIUM	5
Old Dominion University	5
Eastern Virginia Medical School	28
Massachusetts Institute of Technology	34
University of Texas Health Science Center at San Antonio (UTHSCSA)	41
Washington University at St. Louis	44
University of Wisconsin	49
PURDUE CONSORTIUM	54
Purdue University Calumet	70
University of Arkansas at Little Rock (Since January 1, 2006)	85
Northwestern University	87
MURI DIRECTORY	88
ADDENDUM (Review Paper)	90

## OLD DOMINION UNIVERSITY CONSORTIUM

### Old Dominion University

#### I. ACHIEVEMENTS

Subcellular effects of radiofrequency radiation was the topic of the Multidisciplinary University Research Initiative (MURI) with scientists from six academic institutions (Old Dominion University, Eastern Virginia Medical School, Massachusetts Institute of Technology, Washington University, University of Wisconsin and University of Texas Health Science Center) participating. Old Dominion University served as the lead university focusing initially on four topics:

- a) **the development of nanosecond pulsed power systems for biological studies**
- b) **the development of imaging methods and exposure systems for *in vitro* studies**
- c) **modeling of nanosecond pulse effects on cells**

The results of the pulsed power research were shared with the teams at MURI universities (including that in a sister MURI, administered by Purdue University). Modeling efforts were coordinated with those at the modeling group at MIT, and exposure systems have been developed in partnership with the team at U. Wisconsin. Finally, the efforts at Old Dominion University have been closely coupled to the basic studies on nanosecond pulse effects performed at Eastern Virginia Medical School.

This interaction among the MURI teams, which is depicted in figure 1, has led to major achievements in the development of pulse generators, novel diagnostic techniques, and most importantly, to an increased understanding of subcellular effects caused by intense nanosecond pulses. In the following, we will focus on the achievements of the team at Old Dominion University. Those of our colleagues in the MURI team can be found in the sections devoted to the individual teams.

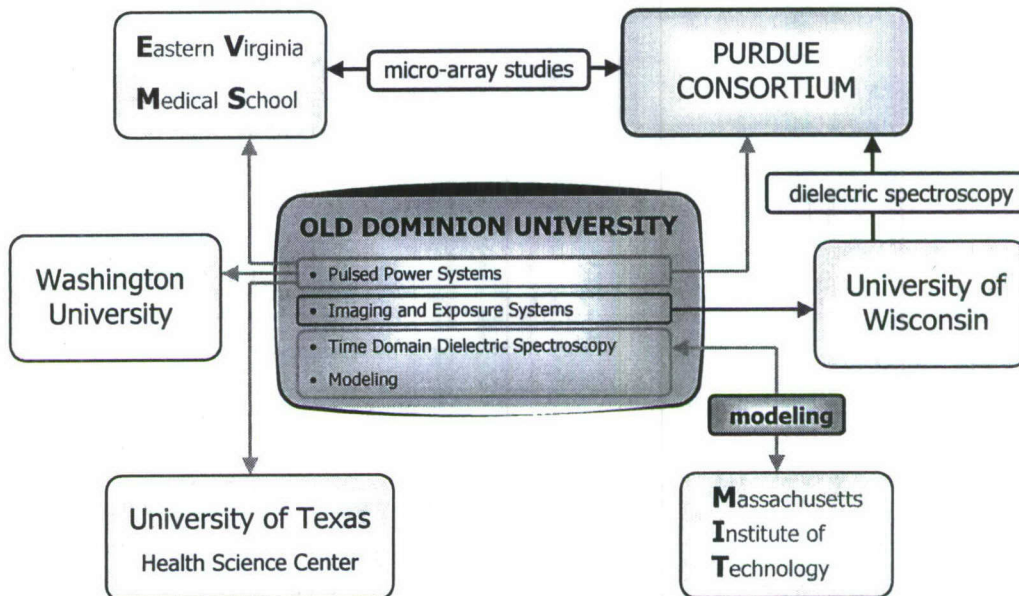


Fig. 1 Interaction between MURI teams

A comprehensive report of the efforts of the MURI, focusing on studies performed at Old Dominion University and Eastern Virginia Medical School, has been published in the *IEEE Transactions on Dielectrics and Electrical Insulation*, Vol. 14, pp. 1088-1109 (2007) and is attached to the final report.

## **a. PULSE GENERATORS FOR BIOELECTRIC STUDIES**

The study of intracellular electro-effects and applications using such effects require electrical pulse generators that provide well-defined high voltage pulses with fast current rise to the load. The load could be either cells in suspension or tissue. The applied voltage is determined by the required threshold electric field for intracellular effects. This value depends strongly on pulse duration: it seems that, to achieve comparable effects, the electric field times the pulse duration must be approximately constant. The onset field for any effect is also strongly dependent on the cell type. For single pulses or pulse trains with only a few pulses, generally up to five, typical electric fields for apoptosis range from tens to hundreds of kV/cm. The highest values for 10 ns pulses were 300 kV/cm. These fields are lower for pulse trains with an extended number of pulses.

In order to study intracellular electro-effects, two types of pulse generators have been used. One was developed for experiments which require large numbers of cells, such as those in which flow cytometry is used, or where studies are performed on tissues. This pulse generator is based on the Blumlein concept and can deliver pulses of 10 ns and up to 40 kV into a 10  $\Omega$  resistive load. This system served as a standard pulse source for a consortium of scientists in the Multi-disciplinary University Research Initiative on "Subcellular effects of narrow band and wideband radiofrequency radiation," and was used for experiments or diagnostic methods.

A second class of pulse generators was designed for observations of individual cells under a microscope. Consequently, the gap distance can be reduced to the 100  $\mu$ m range, and simultaneously, voltage requirements can be relaxed. Instead of typical pulsed power components, low voltage, high frequency cables can be used in the design together with fast semiconductor switches. Such types of pulse generators – micropulsers – operate at voltages of less than one kV. However, because of the small electrode gap, electric field intensities of up to 100 kV/cm are possible and can be applied to cells in suspension that are placed in the electrode gap. This type of pulse generator has been built with variations in pulse length, type of switch, type of pulse forming network, as a line type pulser, and as a hard tube pulser in many of the ultrashort pulse studies. The compactness of these pulse generators makes them standard tools for ultrashort pulse effect applications.

## **b. REAL-TIME (NANOSECOND) IMAGING OF MEMBRANE VOLTAGES**

Using laser stroboscopy, we were able to measure the change in transmembrane voltage with a temporal resolution determined by the laser pulse duration of 5 ns. The transmembrane voltage was quantified by staining the cell membrane with a fluorescent voltage sensitive dye. Changes in fluorescence intensity quantify the change in transmembrane voltage. In order to utilize the short illumination time of the laser, the response of the dye needs to be on the same order of magnitude but faster. Recently, a new voltage sensitive dye, Annine-6, was developed with a voltage-regulated fluorescence response that depends only on the shift of energy levels by Stark-effect. This fast electronic process theoretically enables a sub-nanosecond voltage sensitive response.

To investigate the membrane charging in response to an ultrashort pulsed electric field, we stained Jurkat cells (a T-cell leukemia cell line) with Annine-6 and exposed them for 60 ns to an electric field of 100 kV/cm. At different times during the exposure the cells were illuminated with a laser pulse of 5 ns duration (FWHM). The recorded changes in fluorescence intensity allow us to estimate transmembrane voltages based on calibration curves (which were developed for transmembrane voltages of up to 0.3 V, and extrapolated for our results) and monitor their temporal development.

The results of this study show that an increase in plasma membrane conductivity occurs within a few nanoseconds (less than the temporal resolution of our method), as is indicated by the deviation of the measured voltage from the ideal temporal voltage development that would be expected for a membrane that is considered to be an ideal insulator. After 20 ns, a second increase in membrane conductance is seen, which might be due to the formation of nanopores. The membrane voltage decrease after the pulse

is determined by the discharge of the membrane, but also by the residual applied voltage. The voltage after the 60 ns pulse, does not reach zero immediately.

### **c. MODELING OF THE INTERACTION OF PULSED E-FIELDS WITH CELLS**

Modeling of pulsed electric field effects on the charge distribution in biological cells, performed at ODU and published in numerous papers (see list of publications), ranges from simple analytical models to those generated using molecular dynamics. Molecular dynamics simulations provide the most basic and fundamental approaches for modeling the effect of electric fields on cells. The part of the cell that is to be modeled is considered as a collection of interacting particles. In the case of a cell membrane, which is a lipid bilayer, these particles are DPPC (Dioleoyl-phosphatidyl-choline) molecules that are characterized by charged subgroups. Thus, the structural details on the nanoscale can be included into the simulation for maximum accuracy and relevant physics. For each of the molecules the equation of motion (Newton's equation) is solved, with the force on each charge and dipole within the molecular structure generated by the surrounding charges and dipoles. In spite of its simplicity, this modeling method has only recently been used to model cellular membranes under electrical stress. The simulation difficulties include the immense number of particles that need to be considered, and also the small time steps, both requiring the use of high-speed computer clusters. This makes molecular dynamics a computationally intensive approach. As an example, Joshi, in the ODU team, has used 164,000 particles to model a patch of membrane, with time steps on the order of one femtosecond. This means that modeling small parts of a cell and following the membrane changes numerically can only be done for times on the order of 10-20 ns. Therefore, molecular dynamics, at this point in time, is restricted to modeling small parts of a cell and to ultrashort (ns) pulse effects. The importance of this method is in the visualization of membrane effects on this timescale, the inclusion of complex underlying physics, and the determination of critical electrical fields for membrane changes on the nanoscale, e.g. for pore formation.

This information can be used in less computer intensive, but more comprehensive models (i.e., for describing larger systems, such as an entire cell). In these models the cells are represented as micro resistor-capacitor (RC) units, with the resistive parts being time-dependent. The information from the molecular dynamics (for example, pore densities and hence, the angular dependent membrane resistance) has then been used as the basics to determine the dynamic resistance for such simpler models. They allow us to determine the response of electric fields on the entire cell, including the plasma and subcellular membranes. However, these models still call for extensive numerical efforts when high spatial resolution is required. So, for quick and simple estimates of the pulsed field effects on cells, analytical approaches can be used to describe the critical electric fields for pore formation. Although such models will not provide complete information for complicated structures, they can provide guidance to the experimentalist in choosing an appropriate electric field-pulse duration range for bioelectric effects.

The topics discussed in the previous sections were the major focus of our research at ODU during the first three years of the grant. In the final two and a half years we have focused, in addition, on

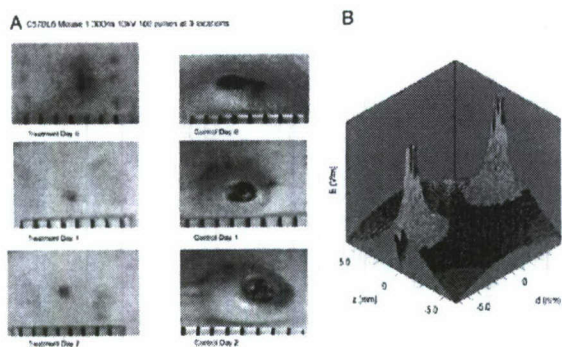
#### **d. applications of ultrashort pulsed effects and**

- 1. treatment of skin cancer**

- 2. advanced wound healing**

#### **e. studies of picosecond pulses with emphasis on development of near field, high electric field antennas for bioelectric applications.**

## d.1. TREATING SKIN CANCER



**Figure 2** Response of melanoma to 3 applications of 100 pulses using a pair of needle electrodes. A. Treated tumor (left) shrinks following pulse application while control tumor (right) continues to grow. B. Electric field distribution for needle electrode during pulse application.

Electric pulses 300 ns in duration and 40 kV/cm can cause total remission of skin cancer in mice. B16-F10 melanoma cells injected into SKH-1 mice generate melanoma tumors that are 3-5 mm in diameter within 4 days following injection. When these tumors are treated with 300 of the pulses just described, they exhibit three rapid changes. 1) The nuclei of the tumor cells begin to shrink rapidly and the average nuclear area falls by 50% within an hour; 2) Blood flow to the tumor stops within a few minutes and does not return for a week or two; 3) The tumor begins to shrink and within two weeks has shrunk by 90%. Most of the roughly 200 tumors treated begin to grow again at this point, but, if treated a second time, will completely disappear. We have begun a long-term study in

which we are following animals for 120 days beyond the date when their tumors were no longer visible. Thus far all 18 animals in the treated group exhibit complete remission with no recurrence after 4 months while all 18 control tumors either grew to a size that required that we euthanize the animal or are still present on the animal.

The pulses were applied with two different electrode types. We started with needle electrodes that penetrated the skin (Figure 2) and then began using parallel plate electrodes in a clothespin configuration. Parallel plate electrodes have the advantage that all of the tissue placed between the electrodes is exposed to the same electric field, in contrast to needle electrodes, in which the field is much higher near the electrode than far from it.

These nsPEF stimulate murine melanomas to self-destruct. This is a novel and deadly cellular response to this new nanosecond time domain of pulsed electric field application. While we have not yet tested this technique on humans, it is likely that it may have advantages over the surgical removal of skin lesions because incisions through the dermis often leave scarring on the healed skin. nsPEF kills the tumor without disrupting the dermis so that scarring is less likely. It should also be effective on other tumor types located deeper in the body, if a catheter electrode can be guided to the tumor. Among its most intriguing characteristics is the incredibly short time that these cells have been exposed to the electric field. All of the tumor regression shown here resulted from a total electric field exposure time of 120  $\mu$ s or less. A second important characteristic is the low energy delivered to the tissue. Each 300 ns pulse of 40 kV/cm delivers only 0.18 J to the tissue between the 5 mm plates. Based on the specific heat capacity of water, this would increase in tissue temperature by only 2-4  $^{\circ}$ C and our measurements of the tumor temperature during treatment confirms this. Two seconds between pulses provides more than adequate time for this heat to dissipate so that the tissue will not heat up beyond this. This highly localized and drug-free physical technique offers a promising new therapy for tumor treatment.

## d.2. PLATELET AGGREGATION AND GROWTH FACTOR RELEASE: A STEP TOWARDS ACCELERATED WOUND HEALING

It has been shown that nanosecond pulses have a similar effect on platelets as thrombin, an agonist that promotes aggregation. This pulsed aggregation process is initiated by the release of calcium from internal stores. This is consistent with the well-known fact that aggregation of platelets by known agonists, such as thrombin, requires an increase in intracellular free calcium. The data obtained with nsPEF on calcium mobilization and calcium influx is reminiscent of the well-known capacitative or store-operated calcium

entry process induced by hormones (e.g. thrombin) in many non-excitable cells. In this process, when intracellular calcium is mobilized from the ER, there is an activation of an influx process in the plasma membrane, i.e. the two processes are coupled.

In addition to aggregation, an increase in PDGF (platelet derived growth factor) released from washed platelets has been observed after pulsing with nanosecond pulses. This release is most likely also due to subcellular electromanipulation. Platelets are rich in alpha granule growth factors (i.e. platelet derived growth factor, PDGF, transforming growth factor beta, TGF- $\beta$ ). Release of this growth factor is essential for wound healing.

This concept of using nanosecond pulsed electric fields for wound healing is exciting for several reasons. First, activation of platelets using nsPEF will provide the medical community with an alternative to the use of thrombin (which has been associated with allergic reactions in some patients). Secondly, nsPEF provides a focused, localized stimulus for platelet activation, whereas, other platelet activators such as thrombin, adenosine diphosphate (ADP), thromboxane or collagen can all enter the circulation and have systemic effects.

#### **e. SUBNANOSECOND PULSE EFFECTS – A STEP TOWARDS USING ANTENNAS AS PULSE DELIVERY SYSTEMS**

Reducing the pulse duration of intense electric field pulses from nanoseconds into the subnanosecond range will allow us to use wideband antennas to deliver the electromagnetic fields into tissue with a spatial resolution in the centimeter range. In order to explore the biological effect of intense, subnanosecond pulses, we have developed a generator which provides voltage pulses of 160 kV amplitude, 200 ps rise-time, and 800 ps pulse width. The pulses are delivered to a cylindrical Teflon® chamber with polished flat electrodes at either end. The distance between the electrodes is variable and allows us to generate electric fields of up to 1 MV/cm in cell suspensions. The pulses have been applied to B16 (murine melanoma) cells, and the plasma membrane integrity was studied by means of trypan blue exclusion. For pulse amplitudes of 550 kV/cm, approximately 50% of the cells took up trypan blue right after pulsing, whereas only 20% were taking it up after one hour. This indicates that the plasma membrane in a majority of the cells affected by the pulses recovers with a time constant of about 1 hour. The cells which show trypan blue uptake after this time are suffering cell death through apoptosis. Evaluation of the experimental results and molecular dynamics modeling results indicate that with a pulse duration of 800 ps, membrane charging and nanopore formation are the dominant bioelectric effects on B16 cells.

## **II. Personnel, Publications, Patents**

### **A. Old Dominion University, Personnel Supported**

#### **Karl H. Schoenbach, Professor, PI**

Juergen Kolb, Research Assistant Professor

Uwe Pliquet, Research Associate Professor

Nianyong Chen, Post Doctoral Researcher

J. Thomas Camp, PhD Candidate

Xinhua Chen, PhD Candidate

Wentia Ford, PhD Candidate

Allen Garner, PhD, Graduated

Emily Hall, PhD, Graduated

Wei Ren, PhD Candidate  
 Jody White, PhD, Graduated, Postdoctoral researcher  
 Jie Zhuang, PhD Candidate  
 W. Hunter Baldwin, MS Candidate  
 Shaka S. Scarlett, MS, Graduated  
 Rachel Shevin, MS, Graduated  
 Sridar Babu Talla, MS Candidate (left in Fall of 2004 to accept a position at a company)  
 Robert Price, Undergraduate/MS Candidate  
 Jing Yang, BS, Graduated  
 David Anderson, Technician

**Ravindra Joshi, ODU, Co-Principal Investigator**

Qin Hu, Ph.D., Graduated  
 Sridhara Viswanadham, Ph.D., Graduated  
 Jiahui Song, Ph.D. graduate student  
 Guogang Zhao Ph.D. Graduated  
 Anh Nguyen, MS graduate student

**B. Publications and Presentations**

(due to the close interactions between the groups, the Schoenbach and Joshi reports are combined)

**PUBLICATIONS IN REFEREED JOURNALS**

Karl H. Schoenbach, Shu Xiao, Ravindra P. Joshi, J. Thomas Camp, Tammo Heeren, Juergen F. Kolb, and Stephen J. Beebe, "The Effect of Intense Subnanosecond Electrical Pulses on Biological Cells," submitted to IEEE Trans. Plasma Science

R.P. Joshi, A. Mishra, J. Song, A. Pakhomov, and K.H. Schoenbach, "Simulation Studies of Ultra-Short, High-Intensity Electric Pulse Induced Action Potential Block in Whole-Animal Nerve Segments", to appear in IEEE Trans. Biomedical Engineering

J. Zhang, P.F. Blackmore, B.Y. Hargrave, S. Xiao, S.J. Beebe, and K.H. Schoenbach, "The Characteristics of Nanosecond Pulsed Electrical Field Stimulation on Platelet Aggregation in Vitro," submitted to J. Bioelectrochemistry.

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K.H. Schoenbach, "Bioelectrics - Using Nanosecond Pulsed Power Technology to Control Biological Cell Functions," Peter Haas Award Address, Plenary Talk, 2007 IEEE Pulsed Power and Plasma Science Conference, Albuquerque, NM, June 22, 2007.

K.H. Schoenbach, S. Xiao, J.T. Camp, T. Heeren, J.F. Kolb, J.A. White, M. Migliaccio, R.P. Joshi, and R. Nuccitelli, "From Submicrosecond To Subnanosecond Pulses – Entering a New Domain of Electric Field–Cell Interactions," 2007 IEEE Pulsed Power and Plasma Science Conference, Albuquerque, NM, June 20, 2007

Juergen F. Kolb, Jody A. White, Wolfgang Frey, Shaka Scarlett, Rachael Shevin, Andrei Pakhomov, Stephen J. Beebe, E. Steve Buescher, Peter F. Blackmore, Ravindra P. Joshi, Richard Nuccitelli, Karl H. Schoenbach, "Immediate, Non-Physiological Responses of Mammalian Cells to Nanosecond Pulsed Electric Fields," Spring Meeting Deutsche Physikalische Gesellschaft (DPG), March 19-23, 2007, Duesseldorf, Germany.

Karl H. Schoenbach, "From Submicrosecond to Subnanosecond Pulses – Entering a New Domain of Electric Field – Cell Interactions," Keynote Address, Intern. Workshop on Bioelectrics, Kumamoto University, Kumamoto City, Japan, February 7, 2007.

K.H. Schoenbach, "Biological Effects of Nanosecond Electrical Pulses," Engineering Colloquium, Norfolk State University, Norfolk, VA, October 6, 2006.

K.H. Schoenbach, "Subcellular Effects of Nanosecond Electric Pulses," University of Erlangen, Germany, Department of Physics, August 2, 2006.

K.H. Schoenbach, "Biological Effects of Nanosecond Electrical Pulses and Medical Applications," Medical University, Urumqi, China, June 14, 2006.

K.H. Schoenbach, "Subcellular Effects of Nanosecond Pulses," Institute of Electrical Engineering, Chinese Academy of Sciences, Beijing, China, June 9, 2006.

K.H. Schoenbach, "Biological Effects of Nanosecond Electrical Pulses," Peking University, Beijing, China, June 7, 2006.

K.H. Schoenbach, "Bioeffects – Overview," Biological Effects of ElectroMagnetics (BEEM) Workshop, University of Missouri-Columbia, February 20, 2006.

K.H. Schoenbach, "Intense Ultrashort Electrical Pulses Manipulate Cellular Organelles," in Symposium on "Electric Forces in Biology and Medicine," at the AAAS Annual Meeting, St. Louis, Missouri, February 18, 2006.

K.H. Schoenbach, "Nanosecond Electrical Pulses Open a Gateway into Biological Cells," College of Engineering & Electrical and Computer Engineering Seminar, Texas Tech University, Lubbock, TX, January 27, 2006.

K.H. Schoenbach, "Subcellular Effects of Nanosecond Electrical Pulses," International Workshop on Bioelectrics, Kumamoto, Japan, November 12, 2005.

K.H. Schoenbach, "Subcellular Effects of Nanosecond Electrical Pulses," IEEE – NPSS Chapter Workshop on Bioelectrics, Forschungszentrum Karlsruhe, Germany, October 17, 2005

K.H. Schoenbach, "Subcellular Effects of Nanosecond Electrical Pulses," Biomedical Engineering Seminar Series, University of Chicago, Argonne National Laboratory, Illinois Institute of Technology, Chicago, May 31, 2005.

K.H. Schoenbach, "Subcellular Effect of Nanosecond Electrical Pulses," Physics Seminar, College of William and Mary, Williamsburg, VA, March 19, 2005.

K.H. Schoenbach, "Subcellular Effects of Nanosecond Electrical Pulses," CNRS - Institut Gustave-Roussy, Paris, France, February 23, 2005, and Institut de Pharmacologie et de Biologie Structurale du CNRS, Toulouse, France, February 24, 2005, and at Ecole Centrale Paris, Paris, France, February 25, 2005.

K.H. Schoenbach, "Nanosecond Electrical Pulses Open a New Gateway into Biological Cells," Physics Seminar, East Carolina University, Greenville, NC, November 12, 2004.

K.H. Schoenbach, "Mechanism of Interaction between Biological Cells and Electric Fields," Workshop on the Effects of Pulsed Electric Fields on Biological Systems, University of Salerno, Italy, September 24, 2004.

K.H. Schoenbach, "Future Trends in PEF Technology and Applications," Workshop on the Effects of Pulsed Electric Fields on Biological Systems, University of Salerno, Italy, September 24, 2004.

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K.H. Schoenbach, "Ultrashort Electrical Pulses Open a New Gateway into Cells," Plenary Talk, 26<sup>th</sup> Annual Meeting of the Bioelectromagnetics Society, Washington, DC, June 21, 2004.

K.H. Schoenbach, "Ultrashort Electrical Pulses Open a New Gateway into Biological Cells," Peking University, Beijing, China, June 7, 2004.

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K.H. Schoenbach, R.P. Joshi, S.J. Beebe, and E.S. Buescher, "Ultrashort Electrical Pulses Open a New Gateway into Biological Cells," Workshop on High Field Effects and fast Responses in Biosystems, as part of the 2003 IEEE Intern. Conf. on Electrical Insulation and Dielectric Phenomena, Albuquerque, NM, October 19, 2003.

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S.J. Beebe, P.M. Fox, J. White, P.F. Blackmore, L.J. Rec, R.P. Joshi, and K.H. Schoenbach, "Nanosecond Pulsed Electric Fields Modulate Cell Function Through Intracellular Signal Transduction Mechanisms," 17<sup>th</sup> Intern. Symposium on Bioelectrochemistry and Bioenergetics, Florence, Italy, June 24, 2003.

Karl H. Schoenbach, R. Joshi, S. Beebe, and S. Buescher, "Bioelectrics," Research Priorities in the Life Sciences Conference, Boar's Head Inn in Charlottesville, Virginia, May 28, 2003.

Karl H. Schoenbach, R. Joshi, E.S. Buescher, and S. Beebe, "Ultrashort Electrical Pulses Open a New Gateway into Cells," Plenary Talk, 38<sup>th</sup> Winter Seminar on Biophysical Chemistry, Molecular Biology and Cybernetics of Cell Functions, January 27, 2003, Klosters, Switzerland.

Karl H. Schoenbach, "Biological and Medical Applications of Pulsed Electric Fields," Seminar, College of Engineering, University of Michigan, Ann Arbor, December 13, 2002.

Karl H. Schoenbach, "Selective Permeabilization of Intracellular Organelles," Gordon Research Conference on Bioelectrochemistry, Mount Holyoke College, South Hadley, MA, July 22, 2002

Karl H. Schoenbach, Stephen Beebe, and Stephen Buescher, "Electromedical Applications of Pulsed Power," Power Modulator Conference, Workshop Session 2: Biological Applications, Hollywood, CA, July 3, 2002

#### D. Patents and Patent Applications

Invention	Serial number	Country Filed	Inventors
Appartus for generating electrical pulses and methods of using the same	4809502.0	Europe	Beebe Schoenbach
Appartus for generating electrical pulses and methods of using the same	2004/023078	PCT US	Beebe Schoenbach
Appartus for generating electrical pulses and methods of using the same	20044277376	Australia	Beebe Schoenbach
Appartus for generating electrical pulses and methods of using the same	2006-521152	Japan	Beebe Schoenbach
Appartus for generating electrical pulses and methods of using the same	10/564,994	PCT US	Beebe Schoenbach
Appartus for generating electrical pulses and methods of using the same	CA 2533116	Canada	Beebe Schoenbach
Activation of Calcium-Mediated Cell Functions In Cells and Tissues, Including Aggregation of Human Platelets, By Nanosecond Pulsed Electric Fields	60/636,695	Prov US	Blackmore, Beebe, Buescher, Schoenbach
Activation of Calcium-Mediated Cell Functions In Cells and Tissues, Including Aggregation of Human Platelets, By Nanosecond Pulsed Electric Fields	11/305,121	Utility appl	Blackmore, Beebe, Buescher, Schoenbach
Ultrawide Band Antenna For Near-Field Operations in Tissue	60/709,527	Prov US	Schoenbach, Beebe
Ultrawide Band Antenna For Near-Field Operations in Tissue	60/800,729	Prov US	Schoenbach, Beebe
Ultrawide Band Antenna For Near-Field Operations in Tissue	PCT/US2006/0032440	PCT US	Schoenbach, Beebe
Local Hyperthermia with Ultrawide band high power focussing emitters	60/978035		Schoenbach, Baum, Xiao
Wide band Bio Imaging System	61/016,363		Schoenbach, Xiao

## II. Personnel, Publications, Interactions, Patents

### A. Old Dominion University, Personnel Supported (Xu Group)

#### X. Nancy Xu, ODU Co-Principal Investigator

Hongwu Xu, Postdoc

Qian Wan, Ph.D. Candidate

### B. Publications and Presentations

#### PUBLICATIONS IN REFEREED JOURNALS

T. Huang, P. D. Nallathamby, D. Gillet, and X. Nancy Xu\*, "Design and Synthesis of Single Nanoparticle Optical Biosensors for Imaging and Characterization of Single Receptor Molecules on Single Living Cells", *Anal. Chem.* **79**, 7708-7718 (2007).

X. Nancy Xu\*, "New Frontiers in Ultrasensitive Bioanalysis: Advanced Analytical Chemistry Applications in Nanobiotechnology, Single Molecule Detection, and Single Cell Analysis", a Wiley Chemical Analysis Series, 2007 (invited).

H. Xu, P. D. Nallathamby, X. Nancy Xu\*, "Real-time Imaging of Subcellular Structures and Membrane Transport Kinetics of Single Human Leukemic Cells Induced by Nanosecond Electric Pulses" (submitted).

#### PUBLICATIONS IN CONFERENCE PROCEEDINGS AND BOOK CHAPTERS

X. Nancy Xu\*, W. Brownlow, Q. Wan, J. Kolb, and K. H. Schoenbach "Real-time imaging of effects of electric fields on subcellular structures using nanoparticle optics and single live cell microscopy", *3rd ElectroMed Conference in San Antonio, TX* (June 2003) (Platform presentation).

X. Nancy Xu\*, Q. Wan, W. Brownlow, J. Kolb, and K. Schoenbach, "Real-time study of effects of electric fields on binding of single ligand-receptor interaction on living cell surfaces", *3rd ElectroMed Conference in San Antonio, TX* (June 2003) (Poster).

X. Nancy Xu\*, Q. Wan, J. Kolb and K. Schoenbach, "Real-time monitoring of effects of electric fields on kinetics of membrane transport in single living cells", *Proc. of the 26th Annual Meeting of The Bioelectromagnetics Society* (June 2004) (Platform Presentation).

Q. Wan, X. Nancy Xu\*, J. Kolb and K. Schoenbach, "Real-time study of accumulation effects of electric fields upon membrane transport in single living cells", *Proc. of the 26th Annual Meeting of The Bioelectromagnetics Society* (June 2004) (Poster).

Q. Wan, X. Nancy Xu\*, J. Kolb, and K. Schoenbach "Real-time probing of the effect of high electric fields on sub-cellular functions", *Proc. of the 2004 Pittsburgh Conference*.

Q. Wan and X. Nancy Xu\*, "A novel technique for real-time investigation of electric-field effects upon subcellular function in single living cells" *Proc. of the 2005 Pittsburgh Conference* (Oral Presentation; ABS N0 1040-4).

Q. Wan and **X. Nancy Xu\***, "Real-time tuning membrane transport of single living cells using electric fields" *Proc. of the 2005 Pittsburgh Conference* (Oral Presentation; ABS No. 1430-6).

Q. Wan, X. and Nancy Xu\*, "Real-time monitoring of subcellular mechanism induced by electric fields" *Proc. of the 2005 Pittsburgh Conference* (Oral Presentation; ABS No. 1430-9).

M. Khalid, C. Q. Zhou\*, A. Bassi, H. L. Gerber, C. C. Tseng, Q. Wan, **X. Nancy Xu\***, "Heat Transfer Analysis of Cell Culture in a Microchannel-Based nsPEF System", Symposium Record Abstracts, ElectroMed2005, Portland, OR, May 2005, p 4 (Oral Presentation).

Q. Wan and **X. Nancy Xu\***, "Timing Intracellular Function of Single Living Cells Using Nanosecond Electric Pulses", Symposium Record Abstracts, ElectroMed2005, Portland, OR, May 2005, p. 25 (Oral Presentation).

Q. Wan and **X. Nancy Xu\***, "Tuning Subcellular Structures and Molecular Interactions of Single Living Cells Using Nanosecond Electric Pulses", Symposium Record Abstracts, ElectroMed2005, Portland, OR, May 2005, p. 131 (Poster Presentation).

**X. Nancy Xu\***, H. Xu, P. Nallathamby, W. Brownlow, "Real-time Tuning Membrane Transport and Subcellular Structure of Single Living Cell Using Electric Fields", *Proc. of the 2006 Pittsburgh Conference*, Orlando, Florida, March 12-17, 2006. (Oral Presentation and ABS No. 1420-3)

## Eastern Virginia Medical School

### I. Achievements

#### **Effects of high intensity, nanosecond pulsed electric fields (nsPEFs) on mammalian cell fate: Effects above and below the threshold for cell death.**

The primary objective of the Multi-University Research Initiatives was to observe and characterize the effects of broad-band radio frequencies on biological cells. As studies progressed a major objective developed to analyze effects of nsPEFs on apoptosis induction using human Jurkat cells, T lymphocyte-like cells, or HL-60 cells, undifferentiated neutrophils, as models for the studies. Apoptosis is a form of programmed cell death that brings cell demise without inflammation, pain and scarring. It is altruistic cell suicide that eliminates cells that are senescent, damaged, diseased or not needed such that surrounding cells are spared the damage to inflammation. During apoptosis cells undergo an orchestrated disassembly of cell structure and function by proteases called caspases. The apoptotic cells are phagocytized by other cells such that the cell raw materials can be recycled. Apoptosis is the antithesis of death by necrosis, where cell membrane integrity fails and cells leak their contents into the surrounding environment leading to inflammation, pain and scarring. In the process of defining nsPEF conditions for these studies, it became clear that there were significant cellular effects that were lethal and non lethal. From these studies analyses were carried out on cell effects that were above and below the threshold for cell death (apoptosis).

A hypothesis was developed that nsPEFs acted on cells as a non-ligand agonist, mimicking the effects of hormones or extracellular chemical on cells. These effects modified cell signal transduction determining cell fate. For effects on cell fate that were below the death threshold, calcium mobilization and growth factor release was the focus, which are outlined below. The results from these studies led to ongoing studies for the potential application to control bleeding and promote healing of wounds. Other possible applications from these results are now focused on roles for calcium mobilization to initiate and modulate neurotransmitter release from excitable cells. This includes potentials to modulate functions at the neuromuscular junction for immobilization and at nociceptive receptors to control pain. For effects that are above the threshold for death, apoptosis induction was the focus as a means to control diseases that involve maladaptive apoptosis functions, such as diabetes, Alzheimers disease and cancer. The primary focus of these studies was on cancer control.

#### **NsPEF effects that are below the threshold for cell death:**

Selective effects on intracellular membranes: One of the earliest hypotheses proposed that, unlike conventional electroporation, nsPEFs had specific effects on intracellular membranes. This turned out to be true and was first demonstrated by Schoenbach et al. (2001) using human eosinophils. It was shown that intracellular vesicles were breached allowing bright green fluorescence from calcein to enter the vesicles from the cytoplasm. These fluorescent vesicles were called "sparklers". The plasma membrane remained relatively intact since cells did not take up red fluorescence from propidium iodide. This demonstrated an effect of nsPEF on porated eosinophil vesicles but not the plasma membrane.

NsPEF-induced PI uptake: direct and indirect electric field effects?

Initial experiments utilized fluorescence microscopy approaches to examine nsPEF effects on propidium iodide uptake which showed that uptake was pulse duration and intensity dependent, and that uptake could occur immediately post-nsPEF application (direct effects), or could be delayed for seconds-minutes after pulse application (indirect effects). In addition, the level of uptake could vary from cell to cell. These observations suggested that nsPEF applications could produce surface membrane permeabilization, could trigger intracellular processes that resulted in surface membrane permeabilization, and that nsPEF-

produced or triggered membrane permeabilization was reversible in some situations (Deng et al., 2003; Buescher and Schoenbach 2003). Similar results were observed in HCT 116 colon carcinoma cells (Hall et al., 2005)

NsPEFs modulate chemotaxis and locomotion in human polymorphonuclear leukocytes (PMNs): Investigation of the nsPEF effects on PMN chemotaxis using real-time microscopy of motile cells demonstrated that the orientation of the applied nsPEF modified the locomotory sensing of the PMN (i.e., the direction of locomotion within a chemoattractant gradient could be modified by an applied nsPEF), and that nsPEF intensity/duration impacted the type and duration of the PMN locomotive response to the applied pulse (Buescher et al., 2004)

NsPEF effects show cell type specificity: Studies using propidium iodide uptake addressed whether nsPEF-produced or triggered effects were universal or had specificity for different cell types. Cell-type specific effects were documented (Hair et al., 2003; Beebe et al., 2004)

NsPEFs induce release of intracellular calcium and activate store operated plasma membrane channels and nanopores: At nsPEF conditions that are below the threshold for cell death calcium is mobilized from intracellular storage sites. HL-60 cells treated with nsPEFs in the absence of calcium were shown release calcium specifically from the endoplasmic reticulum while the plasma membrane remained intact. When calcium was subsequently added to the extracellular media, calcium entered the cells through store operated channels and nanopores in the plasma membrane. This response mimicked the response of HL-60 cells to purinergic agonists, which modulate responses to pain. Using Fura-2, the intracellular calcium changes were quantified. Thus nsPEFs mimicked the actions of a natural agonist on calcium mobilization

NsPEFs induce calcium mobilization in human polymorphonuclear leukocytes (PMNs) suppress locomotion: Evaluating calcium mobilization in greater detail, microscopy-based experiments were performed to determine if this effect allowed intracellular entry of physiologically divalent cations, such as calcium into PMNs, a cell type whose functionality is heavily dependent on intracellular free calcium-based signaling. NsPEF applications were documented to produce rapidly occurring, transient rises in intracellular free calcium in PMNs. NsPEF-produced intracellular free calcium transients that were nsPEF dose and duration-related, resulted from influx of extracellular calcium, and resulted in prompt suppression of PMN function (locomotion) (Buescher et al., 2004a, 2004b);

NsPEFs induce release of calcium and growth factors: In another study Zhang et al., (2007) showed that nsPEFs induced intracellular release of calcium from human platelets. This calcium mobilization resulted in platelet activation and aggregation. Here the calcium was not released from the endoplasmic reticulum, but from some other storage site, most likely alpha-granules. Once again the calcium release was quantified using fura-2. Human platelets also released a growth factor accurately called platelet-derived growth factor. This again demonstrated nsPEF-induced release of molecules from intracellular sites.

Summary of nsPEFs effects on calcium. It appears that cells calcium mobilization in response to nsPEFs is cell type specific. In HL-60 cells and Jurkat cells (unpublished), calcium is initially released from the endoplasmic reticulum while in human platelets it is released from a different storage site, likely alpha granules. In human PMNs, calcium enters the cells from the extracellular media. It is likely that calcium enters cells from the outside through nanopores (all cells tested) and/or through store operated channels (HL-60 and platelets).

NsPEFs have differential effects on cells in the S-phase of the cell cycle: Human HCT 116 colon carcinoma cells were treated with nsPEFs that were at the threshold for cell death induction (Hall et al., 2007). In this study, HCT116 cells were synchronized to S-phase or remained un-synchronized. Unlike un-synchronized cells, S-phase cells under limiting conditions exhibited greater membrane integrity and caspase activation and maintained cytoskeletal structure. In unsynchronized cells, the cytoskeletal structure was re-arranged, exhibiting random organization. Regardless of synchronization, cells exposed to nsPEFs under these conditions primarily survived, but exhibited some turnover and delayed proliferation in cell populations, as well as reversible increases in phosphatidylserine externalization, membrane integrity, and nuclei size. These results show that nsPEFs can act as a non-ligand agonist to

modulate plasma membrane and intracellular structures and functions, as well as differentially affect cells in S-phase, but without effect on cell survival. Furthermore, nsPEF effects on the nucleus and cytoskeleton may provide synergistic therapeutic actions with other agents, such as ionizing radiation or chemotherapeutics that affect these same structures.

NsPEFs penetrate the nucleus and enhance speckle formation: In another study nsPEFs generated nanopores in the interior membranes of cells and modulated cellular functions (Chen et al., 2007). Here, confocal microscopy and flow cytometry were used to observe nuclear speckles, known as small nuclear ribonucleoprotein particles (snRNPs) in Jurkat cells following non-lethal nsPEF conditions. Changes were observed in nuclear speckle labeling that suggested a disruption of pre-messenger RNA splicing mechanisms. Pulse exposure increased the nuclear speckled substructures by approximately 2.5-fold above basal levels while the propidium iodide (PI) uptake in pulsed cells was unchanged. The resulting nuclear speckle changes were also cell cycle dependent. These findings suggest that sub-lethal nsPEFs directly influenced nuclear processes.

NsPEFs induced changes in cellular dielectric properties: The interactions of nsPEFs with biological cells depend critically on the electrical properties of the cells being pulsed (Garner et al., 2007). Time domain dielectric spectroscopy was used to measure the dielectric properties of Jurkat cells before and after exposure to non-lethal nsPEF conditions. The cytoplasm and nucleoplasm conductivities decreased dramatically following pulsing, corresponding to previously observed rises in cell suspension conductivity. This suggests that electroporation occurred, resulting in ion transport from the cell's interior to the exterior. A delayed decrease in cell membrane conductivity after the nsPEFs possibly suggests long-term ion channel damage or use dependence due to repeated membrane charging and discharging. This data is useful in models describing the nsPEF phenomena.

Nanosecond pulsed electric fields (nsPEF) induce direct electric field effects and biological effects on human colon carcinoma cells. A series of experiments was carried out using human HCT colon carcinoma cells that expressed or did not express p53 (Hall et al., 2005). P53 plays a major role in cell responses to DNA damage. These results indicate that nsPEF effects on HCT116 cells include (1) apparent direct electric field effects, (2) biological effects that are p53-dependent and p53-independent, (3) actions on mechanisms that originate at the plasma membranes and at intracellular structures, and (4) an apparent p53 protective effect. These studies demonstrated that nsPEF applications provide a means to explore intracellular structures and functions that can reveal mechanisms in health and disease.

#### **NsPEF effects that are above the threshold for cell death:**

In the beginning of studies that analyzed the effects of ultrashort pulsed electric fields (nsPEFs) on biological cells, scientists at Eastern Virginia Medical School and Old Dominion University were poised to test the hypothesis that these high intensity, nanosecond pulsed electric fields (nsPEFs) would induce apoptosis in eukaryotic cells. The EVMS PI had two studies ongoing investigating the roles of the cyclic AMP (cAMP)-dependent protein kinase (cAPK) to slow apoptosis progression in human neutrophils [Parvathenani et al., 1998] and effects of resveratrol to induce apoptosis in human prostate cells. In both studies, several apoptosis markers were analyzed including caspase activation, DNA fragmentation, annexin-V binding to externalized phosphatidylserine on outer leaflet of the plasma membrane, and cell morphology changes that included cell shrinkage, membrane blebbing, and nuclear condensation. These apoptosis markers were used to show that mammalian cells perceived nsPEFs as a stimulus that altered plasma membrane and intracellular structures and functions beyond the call for living.

Nanosecond, high-intensity pulsed electric fields induce apoptosis in human cells. The first demonstration of nsPEF-induced apoptosis was observed in Jurkat and HL-60 cells (Beebe et al., 2002). This was followed with a much more in depth analyses and demonstration of a full range of apoptosis markers in Jurkat and HL-60 cells (Beebe et al., 2003a, 2003b, 2004) As the pulse duration decreased, plasma membrane electroporation decreased and appearances of apoptosis markers were delayed. When nsPEF conditions were increased in electric field, pulse duration and/or pulse number the threshold for cell death was reached in a cell-type dependent manner. NsPEF induced apoptosis within tens of minutes,

depending on the pulse duration. Annexin-V binding, caspase activation, decreased forward light scatter, and cytochrome c release into the cytoplasm were coincident. Apoptosis was caspase- and mitochondria-dependent, but independent of plasma membrane electroporation and thermal changes. The results suggest that with decreasing pulse durations, nsPEF modulate cell signaling from the plasma membrane to intracellular structures and functions. These studies also demonstrated that apoptosis depended on the release of cytochrome c from the mitochondria. Thus, one of two apoptosis pathway was recruited by nsPEFs in Jurkat cells. One possible pathway is through the intrinsic pathway, which responds to intracellular stimuli. The other is through the type II extrinsic pathway, which responds to stimuli through the plasma membrane.

Similar results were observed when HCT116 colon carcinoma cells were exposed to lethal nsPEF conditions (Hall et al., 2007). nsPEFs induced apoptosis in HCT 116 cells with or without p53. Since about 50% of cancer exhibit mutations in p53, these results indicate that nsPEF should be able to kill cancer cells regardless of p53 status in the tumor. Furthermore, these studies indicated that nsPEF induced caspase activation in the absence of cytochrome c release from the mitochondria. This indicates that apoptosis induction by nsPEF in HCT116 cells occurs regardless of the presence or absence of p53 and the type I extrinsic apoptosis pathway was activated first. Later during apoptosis progression, cytochrome c is released from the mitochondria, indicating the activation of a second apoptosis pathway dependent on mitochondria involvement in these cells. Thus, nsPEFs can induce apoptosis through either mitochondria-dependent or -independent pathways.

In more recent experiments (ongoing, unpublished), nsPEFs were shown to induce apoptosis in B16f10 melanoma cells. Cell death is coincident with caspase activation. Increasing the electric field increases the number of cells that express active caspase activity, as opposed to increasing the amount of active caspase in cells. Caspase activation and cell death is independent of cytochrome-c release as well as the release of other factors that are released during mitochondria-dependent apoptosis. Thus, apoptosis in B16 melanoma cells is completely independent of mitochondrial involvement. This nsPEFs induce apoptosis through multiple pathways depending, at least in part, on the cell type. This suggests that nsPEFs should provide a versatile cancer therapeutic using multiple apoptosis pathways to eliminate cancers.

nsPEFs induces apoptosis in tumors and can eliminate tumors *in vivo*. The *in vitro* studies clearly indicated that nsPEFs induced caspase-dependent cell death in every cell type that was tested. It seemed likely that nanopulses could induce apoptosis and reduce tumor size or eliminate tumors *in vivo*. In fact, in some of the earliest experiments it was demonstrated that nsPEF induced apoptosis and reduce fibrosarcoma tumor size in mice (Beebe et al., 2002). These studies demonstrated time- and electric field-dependent caspase activation and DNA fragmentation in fibrosarcoma tumors *ex vivo* and greater than 60% reduction in tumor size *in vivo* with the first dual needle electrode design (Beebe et al., 2002, 2003, 2004). In later studies, nsPEFs eliminated B16f10 melanoma tumors in mice *in vivo*. These studies indicated caspase activation in tumors, suggesting apoptosis, as well as a decrease in blood flow to the tumor. In 18 mice B16f10 melanoma tumors were eliminated for as long as 180 days at the primary injection site.

This new cancer therapeutic modality based on pulse power technology treats cancer in the absence of drugs by generating broadband radio waves as non-ionizing radiation with nanosecond pulsed electric fields that are high in power (gigawatts) and low in energy (millijoules). They can induce apoptosis and cause loss of vascular viability contributing to infarctive tumor death in a temperature-independent manner. The application of nsPEFs is safe and relatively inexpensive. Unlike chemotherapy it does not have systemic side effects and unlike surgery it is minimally invasive and leaves no scars. The therapeutic application of nsPEFs can provide an inexpensive, new, novel, and effective method to the arsenal for cancer treatment strategies.

## **II. Personnel, Publications, Interactions, Patents**

#### **A. Eastern Virginia Medical School, Personnel Supported**

##### **Stephen J. Beebe, PhD, EVMS Principle Investigator**

Peter F. Blackmore, PhD, Co-Investigator

Gary Z. Morris, PhD Candidate

Jody White, PhD Candidate

Emily Hall, PhD Candidate

Wentia Ford, PhD Candidate

Sam Ramlatchen, graduate student

Dara Peterson, Technician

Sandra Anderson, Technician

##### **E. Stephen Buescher, M.D. Co-Investigator**

#### **B. Publications and Presentations**

Schoenbach KH, Beebe SJ, Buescher ES. Intracellular effect of ultrashort electrical pulses. Bioelectromagnetics. 2001 Sep;22(6):440-8

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## Massachusetts Institute of Technology

### I. Achievements

**The challenge to theory and theory-based models.** At the beginning of the MURI project we were confronted with vivid, compelling descriptions of experiments using megavolt per meter pulses on time scales from 10 ns to 300 ns. Intracellular effects of various types were repeatedly found. And it was argued that the cell's outer membrane (plasma membrane, PM) was not altered. The challenge to theory-based models was clear: How could this occur?

Some of the experiments interpreted intracellular effects as reflecting electroporation (EP) of organelle membranes. For example, calcium ions, could be released from the endoplasmic reticulum (ER) through EP pores. At the same time traditional membrane integrity probes such as propidium iodide (PI) and ethidium homodimer were not observed to enter exposed cells, either during or shortly after pulsing. Absence of detectable uptake was interpreted as "no EP of the PM". Further complicating interpretation was the experimental finding that phosphatidylserine (PS) was translocated from the inner leaflet of the PM to the outer leaflet. Traditionally PS translocation has been viewed as an indicator of apoptosis. Indeed, cell death by apoptosis was a major experimental finding. But EP itself can translocate PS, which confounds interpretation.

In contrast, necrotic cell death generally occurs with conventional EP, with biochemical leakage through the PM. Here "conventional EP" means EP due to pulses with rise times, durations, or fall times longer than the PM charging time constant. Both the ultrashort pulses and conventional EP pulses generally cause little heating. In this sense the underlying mechanisms are non-thermal. Some heating is inescapable when pulsing cells, because of the electrically conducting aqueous electrolytes that are the basis of extra- and intracellular media. Instead, the primary, causal interaction must be electrostatic. This suggests conformational changes as candidates. These include conformational changes of molecules or of cell membranes, both the PM and organelle membranes.

**Our approach and results.** Traditional cell models involve passive membranes. By definition "passive" means fixed electrical properties, viz. conductance and capacitance. Passive models therefore cannot explain cell responses that involve EP, because EP by definition involves creation of "pores" in an electroporated membrane. It is now well established that EP involves a dramatic increase in membrane specific conductance, with relatively little change in specific capacitance. Widely used conventional EP uses pulses with durations of microseconds or longer, usually 100 microseconds to 50 milliseconds in duration. The corresponding field magnitudes are about 1 kV/cm to 100 V/cm for typical mammalian cells. Conventional EP pulses cause transmembrane voltages,  $U_m(t)$ , to reach about 1 V before a burst of pore creation creates such a large membrane local conductance that membrane charging is arrested, and  $U_m$  begins to decrease even during a pulse. EP behavior is both non-linear and hysteretic.

We use cell system models based on interconnected passive and active local models. The electrical behavior and extent of poration is shown in Figs. MIT-1 & 2 below, for two very different electrical field pulses. The first pulse causes conventional EP, the second supra-EP, which involves a high density of only small pores in membranes throughout a cell.

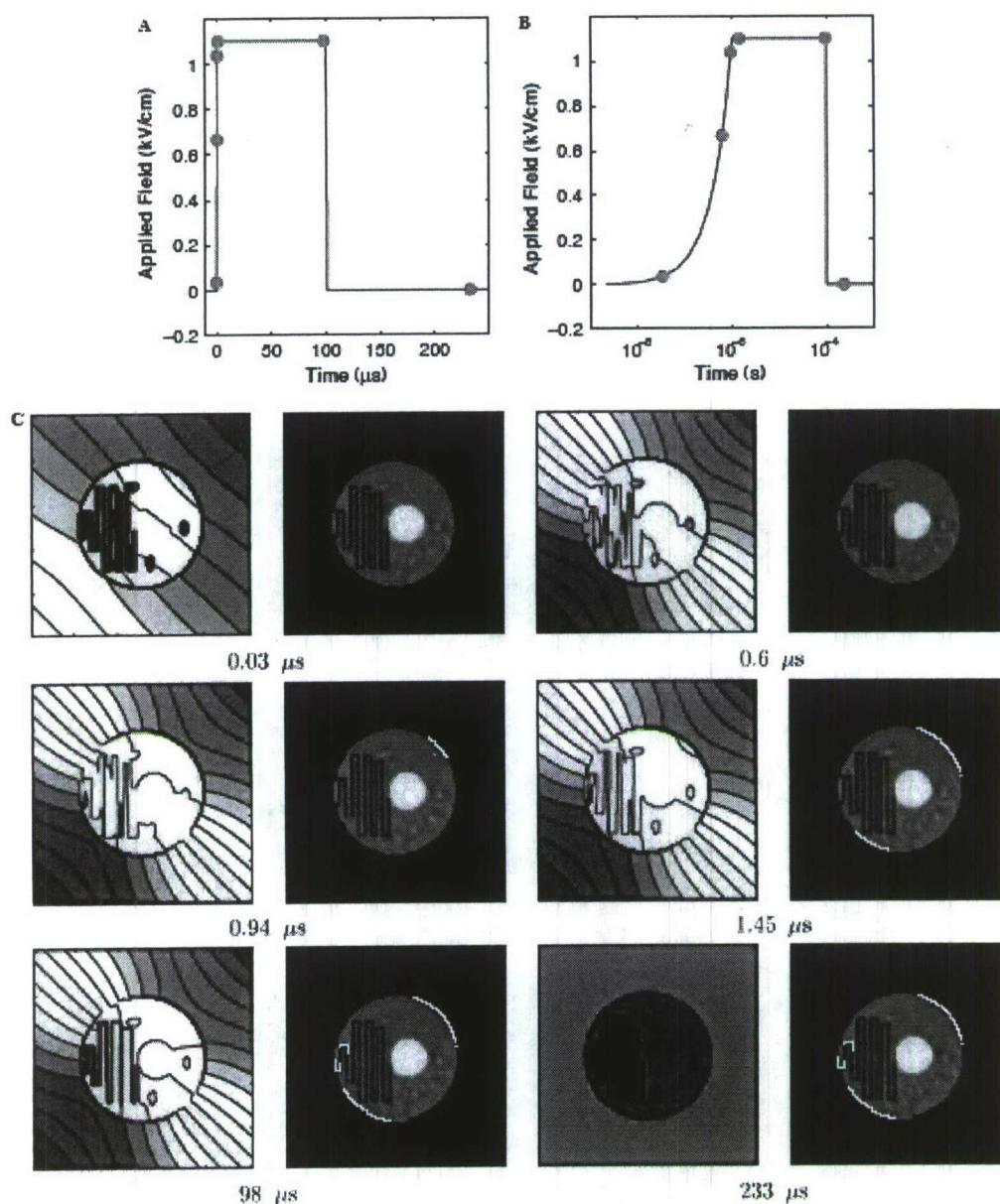


Fig. MIT-1: Electrical and electroporation response of one of our models (Gowrishankar et al. 2006) for a conventional EP pulse (100 microsecond, 1 kV/cm). The upper two panels show the applied field as a function of time on both linear and log time scales, with red dots denoting the times at which the electrical and EP responses are depicted in the bottom six panels. These lower panels show the electrical potential (color) and equipotentials (black curves) in the left panels, while the right panels show the locations of pores (white regions) in the membranes of the cell model. A key feature for conventional EP is the curved equipotentials (field is perpendicular to these curves, so the field mostly goes around the cell). The relatively wide spacings of equipotentials inside the cell indicate a relatively small intracellular field.

Our approach is based on transport lattices, which interconnect local models to form a system model of a cell(s) in two or three dimensions (Gowrishankar & Weaver, 2003). The local models can be simple (e.g. capacitance of a small, local cell membrane) or complex (e.g. local membrane pore creation, expansion, contraction and destruction). We emphasized charge transport in multimembrane systems that represent

the topology of a single, isolated cell with several organelles represented. We have also treated a multicellular system that has fifty irregular shaped cells close together (Gowrishankar & Weaver, 2006).

Recently we have used a meshed transport network to more closely examine the response of a 2D cell model with a circular PM and two different size, asymmetrically located circular organelle membranes to 60 ns, 95 kV/cm pulse. Both a passive membrane version (no local EP model assigned) and an active membrane model (resting potential source and EP models assigned locally) were used (Smith & Weaver, submitted). This model shows that the passive version is inconsistent with experimental and molecular dynamics (MD) results. Complex  $U_m(t)$  behavior is found in the active model, with extensive poration in all membranes. A global depolarization of cell membranes is also predicted, which should persist on a time scale of pore lifetimes (these lifetimes are not yet well established).

Another improved 2D cell model uses a dynamic pore model at the PM. The model shows that some large pores evolve for a conventional EP pulse, and that therefore the PM conductance can increase to create intracellular fields. The electropermeability is computed, and the transport of five small molecules is predicted. The cumulative transport of propidium iodide (PI) is small for a supra-EP pulse, and would not be detected by typical flow cytometry measurements, whereas much larger transport is expected for the conventional EP pulse (Esser et al., submitted).

Overall our results suggest that nanosecond scale, megavolt per meter pulses cause intracellular effects by extensive EP of cellular membranes, transport of small ions and molecules, and post-pulse membrane depolarization that can gate ion channels and pumps.

#### **References not in final report publication list**

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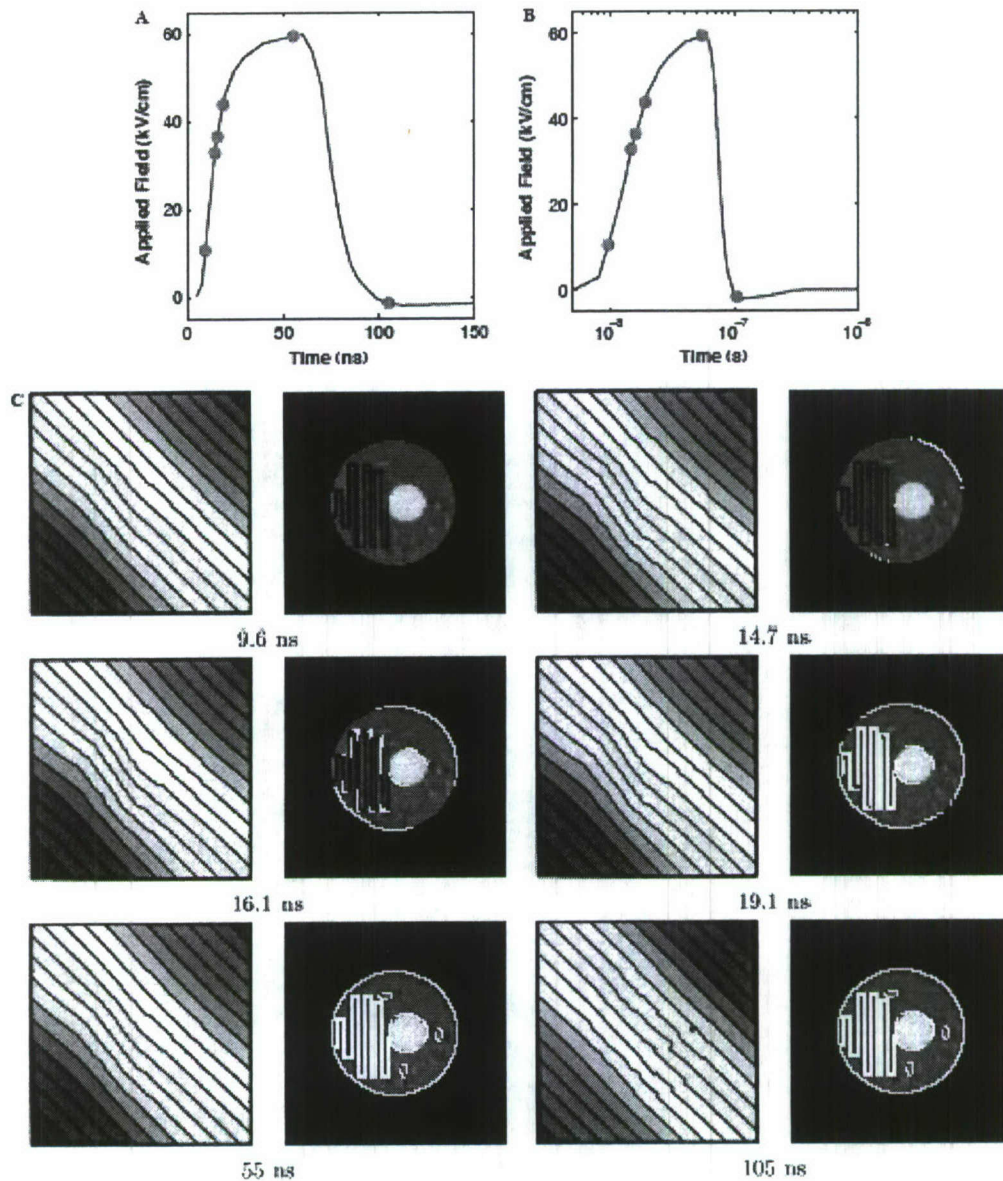


Fig. MIT-2: Response of the model of Fig.-MIT-1 to a supra-electroporating pulse (60 ns, 60 kV/cm; experimental waveform used). Although the model is the same, both the electrical response and the extent of EP are very different. The nearly parallel black equipotential lines only hint at the presence of the cell, with faint ripples indicating the “electrically invisible” cell. The nearly equal equipotential spacing shows that the field is almost the same everywhere, inside and outside the cell. Thus, the field goes through the cell, and organelles experience the field almost as if the PM were not there. The extensive EP (white regions) are sites of highly conducting pores, through which the ubiquitous  $\text{Na}^+$ ,  $\text{K}^+$  and  $\text{Cl}^-$  ions move to hold down  $U_m$ . The maximum  $U_m$  is only 1.5 V (not shown), consistent with experimental observations (Frey et al. 2006). Pores are predicted to be present in large numbers. However, the opportunity for molecular and ionic transport is limited, as small (0.8 nm radius) pores tend to repel charged species because of the Born energy associated with placing charge near the hydrocarbon chains of the membrane’s phospholipids. ATP and other essential molecules may thus be retained, allowing a cell to die by apoptosis rather than necrosis (Gowrishankar et al. 2006, Esser et al., submitted).

## **II. Personnel, Publications, Interactions, Patents**

### **A. Personnel Supported**

#### **James C. Weaver, MIT Principal Investigator**

T. R. Gowrishankar, Research Scientist

Donald A. Stewart, Research Scientist

Axel Esser, Postdoctoral Associate

Zlatko Vasilkoski, Postdoctoral Associate

Kyle C. Smith, Graduate Student

### **B. Publications and Presentations:**

#### **Refereed Journal Publications**

D. A. Stewart, T. R. Gowrishankar and J. C. Weaver (2004) "Transport lattice approach to describing cell electroporation: Use of a local asymptotic model" *IEEE Trans. Plasma Sci.* 32:1696-1708.

D. A. Stewart, T. R. Gowrishankar, K. C. Smith and J. C. Weaver (2005) "Cylindrical cell membranes in uniform applied electric fields: Validation of a transport lattice model" *IEEE Trans. Biomed. Eng.* 52:1643-1653.

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#### **Publications in Conference Proceedings**

T. R. Gowrishankar, D. A. Stewart and J. C. Weaver (2004) "Electroporation of a multicellular system: Asymptotic model analysis" *Proc. 26<sup>th</sup> Ann. Int. Conf. IEEE EMBS, San Francisco, CA*, pp. 5444-5446.

#### **Published Abstracts**

J. Weaver, T. Gowrishankar, D. Stewart, A. Esser, Z. Vaikowski and K. Smith "In silico bioelectromagnetics", BEMS Annual Meeting, Dublin, Ireland, June, 2005.

J. Weaver "Mechanism of nanopore formation in organelle membranes by nanosecond electric pulses" Session on nanosecond electric pulses: A new technology for introducing transient nanopores in organelle membranes and triggering apoptosis. American Society for Cell Biology, 45<sup>th</sup> Annual Meeting, San Francisco, CA December 10, 2005.

J. Weaver "Medical and biological applications of membrane electroporation". AAAS Symposium on Electric Forces in Biology and Medicine, St. Louis, MO February 18, 2006.

T. Gowrishankar, A. Esser, K. Smith and J. Weaver "In silico assessment of nanosecond pulse exposures of cells, tissue and organs", 2006 IEEE Power Modulator Conference, Washington, DC, May 17, 2006.

A. T. Esser, T. R. Gowrishankar, K. C. Smith, W. Kainz, S. J. Seidman and J. C. Weaver "In silico bioelectromagnetics: From molecules and membranes to man" Bioelectromagnetics Society 28<sup>th</sup> Annual Meeting, Cancun, Mexico, June 12, 2006.

A. T. Esser, T. R. Gowrishankar and J. C. Weaver "Asymmetric electroporation and non-equilibrium molecular uptake" Bioelectromagnetics Society 28<sup>th</sup> Annual Meeting, Cancun, Mexico, June 14, 2006.

J. C. Weaver, A. T. Esser, K. C. Smith and T. R. Gowrishankar "Conventional and supra-electroporation: Recent insights" XIX International Symposium on Bioelectrochemistry and Bioenergetics, Toulouse, France, April 2, 2007.

S. M. Kennedy, Z. Ji, J. H. Booske, S. C. Hagness and J. C. Weaver "Experimental and numerical studies of molecular uptake dynamics in HL-60 cells induced by pulsed electric fields" 2007 IEEE Power Modulator Conference, Albuquerque, NM, June 20, 2007.

### **Invited Talks**

J. C. Weaver "Predicting cellular and subcellular responses to strong electric fields" XVII International Symposium of the Bioelectrochemical Society, June 24, 2003, Florence, ITALY.

J. C. Weaver "Transport lattice models of biological systems and supra-electroporation of biological membranes" TAMU-ONR-DARPA Workshop on Quantum Optics, Grand Targhee Resort, Jackson Hole, July 8, 2003, Wyoming, USA.

J. C. Weaver "A model for the induction of cell apoptosis by submicrosecond, megavolt per meter electric field pulses" Bio-Workshop of IEEE CEIDP Conference, October 19, 2003, Albuquerque, NM, USA.

J. C. Weaver "Theory and modeling of cellular response to strong electric field pulses: molecular uptake and intracellular effects" The Society for Physical Regulation in Biology and Medicine, January 8, 2004, San Antonio, TX, USA.

J. C. Weaver, D. A. Stewart and T. R. Gowrishankar "Mechanisms for subcellular responses to ultrashort pulses (intracellular electromanipulation)" Gordon Research Conference on Bioelectrochemistry, New London, CT, USA.

J. C. Weaver "The stochastic nature of electroporation in multi-cellular systems" 26<sup>th</sup> Annual International Conference IEEE EMBS, September 3, 2004, San Francisco, CA, USA.

J. C. Weaver, T. R. Gowrishankar, A. T. Esser, K. C. Smith, D. A. Stewart and Z. Vasilkoski "Transport, sources and sinks: A multiscale, modular approach to modelins systems in biology and medicine" 35<sup>th</sup> Winter Colloquium on the Physics of Quantum Electronics, January 22 – 6, 2005, Snowbird, UT, USA.

J. C. Weaver, A. T. Esser, C. Stewart, Z. Vasilkoski, K. C. Smith and T. R. Gowrishankar "Multicellular, cellular and subcellular fields expected from stun gun exposures" Stun Gun Mini Symposium, University College Dublin, June 19, 2005, Dublin, IRELAND.

J. C. Weaver, T. R. Gowrishankar, D. A. Stewart, A. T. Esser and Z. Vasilkoski "Mechanism of nanopore formation in organelle membranes by nanosecond electric pulses" American Society for Cell Biology, 45th Annual Meeting, December 10, 2005, San Francisco, CA, USA.

J. C. Weaver, A. T. Esser, Kyle C. Smith and T. R. Gowrishankar "In silico assessments of non-lethal interventions" Bioelectromagnetics Society Winter Workshop, February 3, 2006, Phoenix, AZ, USA.

J. C. Weaver "Medical and biological applications of membrane electroporation" AAAS Symposium on Electric Forces in Biology and Medicine, February 18, 2006, St. Louis, MO, USA.

J. C. Weaver, T. R. Gowrishankar, S. K. Burns, A. T. Esser and K. C. Smith "An in silico approach to assessing side effects of HEMI exposures" Aegis Industries Public Safety Workshop on Scientific Factors Associated with Stun Device Injury and Lethality" March 23, 2006, Boston, MA, USA.

J. C. Weaver, T. R. Gowrishankar, A. T. Esser, D. A. Stewart, K. C. Smith and Z. Vasilkoski "An initial approach to in silico bioelectromagnetics for RF exposures" PIERS (Progress in Electromagnetic Research Symposium) 2006, March 27, 2006, Cambridge, MA, USA.

J. C. Weaver "Increasingly realistic cell models" Thirteenth Annual Michaelson Research Conference, August 5, 2006, Freeport, ME, USA.

J. C. Weaver "Modeling biological cells exposed to strong electric fields" Physics Department Colloquium, University of South Florida, November 17, 2006, Tampa, FL, USA.

J. C. Weaver "Ultrashort, high-field electroporation of organelles with cells and tissue – potential applications" Winterseminar, January 24, 2007, Klosters, CH.

J. C. Weaver "BioEM at MIT" IT'IS Foundation ITET Department, Swiss Federal Institute of Technology ETHZ, January 25, 2007, Zurich, CH.

J. C. Weaver "In silico bioelectromagnetics and in vivo biochemical noise" Identification of Research Needs Relating to Potential Biological or Adverse Health Effects of Wireless Communication Devices" National Research Council of the National Academies (by phone), August 8, 2007, Washington DC, USA.

## University of Texas Health Science Center at San Antonio (UTHSCSA)

*Please note: The Principal Investigator of this project at UTHSCSA has retired and we have been unable to reach him at this time – during the preparation of this report. We have therefore included his most recent annual report for the record. For more information please refer to earlier annual reports.*

### I. Annual Accomplishments

The productivity of the UTHSCSA on this very unique and exciting research program, sponsored by the Air Force Office of Scientific Research (AFOSR), was enhanced by cost sharing of personnel by two Departments within the UTHSCSA, the Department of Radiation Oncology and the Department of Biochemistry. Without this collaborative effort, the complex tasks undertaken could not have been accomplished.

#### **The Absence of Direct DNA Damage in the Form of DNA Single-strand Breaks When Human Lymphoblastoid Cells are exposed to Extremely High Peak Power 10 ns Pulsed.**

Participants in the MURI Research Program have been discussing whether or not pulsed 10 ns EMF signals could directly damage DNA. To address this point directly, the following study was performed:

Human lymphoblastoid 244B cells were exposed to extremely high average peak power 10 ns pulses (20 MV/m), which resulted in cell toxicity. The total number of 10 ns pulses was 25 – 100. The cell killing was measured immediately post-exposure, and at 2 hr and 24 hr post-exposure using the trypan blue dye exclusion assay. The cell killing varied with the number of pulses at a given average peak power. For the specific exposure of 25 total pulses, at an average peak pulse height of 200 kV/cm (20 MV/m), the cell viabilities at 2 hr and 24 hr were 73 % and 92 %, respectively. The increase is hypothesized to be due to the loss of dying cells by apoptosis, reported by other MURI laboratories. For DNA single strand break analysis, the cells were exposed at room temperature, using the same exposure protocol as for the viability studies. They were then immediately chilled in a slush ice bath, inhibiting any immediate DNA rejoining repair. The cells were analyzed for DNA single strand breaks using a standard comet assay technique. Slides were examined at 250X magnification using a Zeiss Fluorescent Microscope equipped with a 515-560 nm barrier filter. Image analysis was done using a CCD Pulnix Camera and scored using Komet 5.5 Software. A total of 100 cells per sample (50 cells from two different slides) were examined for DNA migration (tail length in microns), Olive Tail Moment, and % DNA Tail. The results of these studies demonstrated a total absence of evidence for direct DNA single strand breaks immediately after exposure to these unique EMF signals. Any DNA breakage observed must therefore be due to other mechanisms.

#### **Genomic and Proteomic Alterations After Exposure of Human Lymphoblastoid Cells to 10 ns Pulsed EMF.**

Our laboratories have been performing an extensive investigation of genomic and proteomic changes that have been observed to occur after exposure of 244B human lymphoblastoid cells to extremely high peak power (20 MV/m) pulsed electromagnetic fields (PEMFs) with a pulse width of 10 ns. Because of the very large number of variables involved in performing these exposures, and the costs and technical skill needed for the quality performance of the genomic and proteomic assays, the decision was made to investigate the effects of a defined number of pulses. Initially, a total exposure of 25 pulses, at a pulse repetition rate of 1 pulse per 1.5 sec, was chosen. This exposure had been demonstrated to result in readily measurable cell killing, but allowed for the viability of the surviving cells to remain at 70% (when measured at 2 hr post exposure). The logic for the selection was that if we are interested in genomic and

proteomic changes, and their alterations over time, we should be more interested in the changes occurring in a predominantly viable population of cells than in a population of dying or dead cells. The genomic changes observed included both increases and decreases in a wide range of genes at 2 hr and 24 hr post-exposure. The experiments were expanded to include exposures of the cells to 3 or 10 pulses. A range of genomic changes were again observed. The gene changes are being examined using pathway analysis software, to attempt to understand any relationship(s) between the different changes observed. Proteomic changes have also been observed at 2 hr and 4 hr post exposure, and an effort is being made to relate these to the earliest gene changes observed.

### **Curcumin enhances the apoptotic response of nanosecond pulsed electric field**

The high energy ultra short nanosecond pulsed electric field (nsPEF) is a new tool that has the potential to induce different signaling pathways. Previous studies have reported effects of nsPEF on the integrity of intracellular organs, intracellular calcium release, and induction of apoptosis. In the present study the induction of both apoptosis and expression of the anti-apoptotic gene Mcl-1 and c-fos gene expression were observed after 10 nsPEF exposures. The objective of this study was to examine the apoptotic response after nsPEF exposures upon inhibition of Mcl-1 and c-fos expression by curcumin. The studies were performed in 244B human lymphoblastoid cells. The cells were exposed to 10 nsPEF (1, 3, 10, 25 pulses) at average peak field intensity of 200 kV/cm using the pulser device. Loss of mitochondrial membrane potential and active caspases (caspase 9 and 3) was detected following nsPEF exposures, indicating induction of apoptosis. Besides apoptosis induction, an early and dose dependent increase of Mcl-1 and c-fos gene expression was also observed following nsPEF exposures. The nsPEF-induced Mcl-1 and c-fos expression was inhibited in the presence of 50  $\mu$ M curcumin. Upon inhibition of Mcl-1 and c-fos expression by curcumin, the apoptotic response by nsPEF was enhanced. The studies, therefore, indicate that the 10 nsPEF in combination with curcumin might be used as a new tool to modulate apoptosis in cancer treatment.

## **II. Personnel, Publications, Interactions, Patents**

### **A. Personnel Supported**

**Martin L. Meltz, Ph.D, Principal Investigator**

Susan Weintraub, Ph.D, Co-Investigator

Mohan Natarajan, Ph.D, Co-Investigator

Bijaya Nayak, Ph.D.

Norma Vela-Roche, M.S.

Cynthia Galindo, B.S.

Kevin Hakalah, B.S.

### **B. Publications and Presentations**

#### **Published Abstracts**

1. Martin L. Meltz(1), Cynthia Galindo(1), Bijaya Nayak(1), Kevin Hakala(2) and Susan Weintraub(2), and Karl Schoenbach(3), Department of Radiation Oncology (1) and Biochemistry (2), University of Texas Health Science Center at San Antonio, Texas, USA, and the Center for Bioelectrics (3), Old Dominion University, Norfolk, VA

Human Lymphoblastoid Cell Killing by Extremely High Peak Power 10 ns Pulsed EMF Signals is Not Associated With Direct DNA Strand Breakage. The 29th Annual Meeting of the Bioelectromagnetics Society, Kanazawa, Japan June 10-15, 2007

2. Martin L. Meltz(1), Cynthia Galindo(1), Bijaya Nayak(1), Kevin Hakala(2) and Susan Weintraub(2), and Karl Schoenbach(3), Department of Radiation Oncology (1) and Biochemistry (2), University of Texas Health Science Center at San Antonio, Texas, USA, and the Center for Bioelectrics(3), Old Dominion University, Norfolk, VA USA

Genomic and Proteomic Alterations After Exposure of Human 244B Human Lymphoblastoid Cells In Vitro To Extremely High Peak Power 10 ns Pulsed Electromagnetic Fields. The 29th Annual Meeting of the Bioelectromagnetics Society, Kanazawa, Japan June 10-15, 2007

3. Nayak BK, Galindo C, Hakala KW, Natarajan M, Weintraub ST, and Meltz ML. Curcumin enhances the apoptotic response of nanosecond pulsed electric field. The 98th Annual Meeting of American Association for Cancer Research, Los Angeles, California, April 14-18, 2007.

#### **C. Interactions and Activities Related to the MURI Project**

We have taken advantage of the unique proteomic and mass spectrometry expertise of Dr. Susan Weintraub in performing these investigations. We have also taken advantage of the microarray core at the UTHSCSA.

## Washington University at St. Louis

### I. Accomplishments

#### Mathematical Model of Tumor Cell Killing by 10ns 80-300KV Pulses.

As part of our effort to determine if trains of 10 ns high energy electric (HE) pulses induce sufficient cell killing to be considered for the development of therapeutic applications we proceeded to delineate the relationship between cell killing and the combination of electric field strength and pulse number. As a means of quantifying the relationship we developed an empirical mathematical description of the process. The data relation clonogenic cell survival with pulse number showed that there was a true threshold in cell killing, followed by an exponential loss of cell survival with increasing pulse number. Thus the data indicated that low pulse numbers were sublethal or non lethal. However the fact that after a threshold was attained, cell killing was exponential shows that the low pulse numbers are sublethal. This observation is in contrast to the dose response for ionizing radiation in which a quasi-threshold region is followed by exponential cell killing. The key difference is that there is a small probability of cell death in a quasi-threshold, while in a true threshold there is no probability for cell death. The sublethal threshold was shorter at higher voltage pulses. Thus the model needed to show a region of no cell killing followed by a region of exponentially decreasing cell survival.

A successful approach to explaining the threshold began with the energy landscape model proposed in Joshi et al. Physical Review E 69; 2004, which suggests that continued HV pulses reduce the activation energy for the induction of apoptosis. This idea allowed us to postulate a Reducing Energy Barrier Model in which the energy barrier  $D_o$  was a function of pulse number,  $N_p$ , pulse voltage,  $V_p$ , and pulse duration,  $\tau$ . The reducing energy barrier survival equations relate cell killing to pulse number voltage and duration as follows:

$$S = e^{-\tau V_p^2 N_p / D_o}$$

$$D_o = D_M - D_I ((1+b)/(1+be^{-k\tau V_p^2 N_p}))$$

$D_o$  is the activation energy for cell death which is reduced from a maximum of  $D_M$  to a minimum of  $D_M - D_I$ .  $D_M$  is a constant characteristic of the cells.  $D_I$ ,  $k$  and  $b$  are arbitrary constants fit empirically, but are linear functions of pulse voltage, with the function being characteristic for the cell line.

The model fit cell survival data from Dr Meltz's group and ours, covering 7 different voltage values. The model predicted a change in the energy barrier that could be related in first approximation to an increase in mitochondrial membrane potential prior to a breakdown and loss of potential.

#### Effect of High Energy 10 ns Pulses on Cell Size Distribution, Cell Viability and Clonogenic Survival, Apoptosis, and Mitochondrial Membrane Potential.

The clonogenic cell survival curves resulting from the application of trains of high energy, 10 ns pulses (HE) resemble those obtain from X-ray exposure. There is an apparent shoulder followed by a region where the cell killing is an exponential function of the applied dose. The shoulder for the X-ray curve is due to sublethal repair of DNA damage, but this mechanism is not applicable to application of the HE pulses. The purpose of the experiments described below was to investigate the nature of the repairable sublethal damage indicated by the shoulder of the HE clonogenic survival curves.

HeLa cells were exposed to trains of HE pulses ranging from 0 to 600 pulses. After pulsing the cells were counted, viability determined and cell diameter distribution measured using a Vi-cell viability instrument (Beckman-Coulter). Cells were plated for clonogenic survival assay. Examples of the cell size distributions following various numbers of pulses are shown in Figure 1. The mean diameters of these distributions are plotted against the number of applied pulses in Figure 2 and the standard deviations are plotted in Figure 3. Data from previous experiments are also shown. A new thaw of Hela cells was started in 2007 because of a micoplasm contamination. The cell viabilities corresponding to the distributions in Figure 1 are shown in Figure 4 and the corresponding clonogenic survival curves in Figure 5.

The cell size distributions in Figure 1 show clearly that the HE pulses affect the cells at the lowest applied value, and the results in Figure 3 show that the spread of the cell diameters continues to increase with the number of applied pulses. The cell viability, however, is not affected until 300 or more pulses are applied (Figure 4). If the increase in cell diameter distribution below the threshold is a result of cell membrane

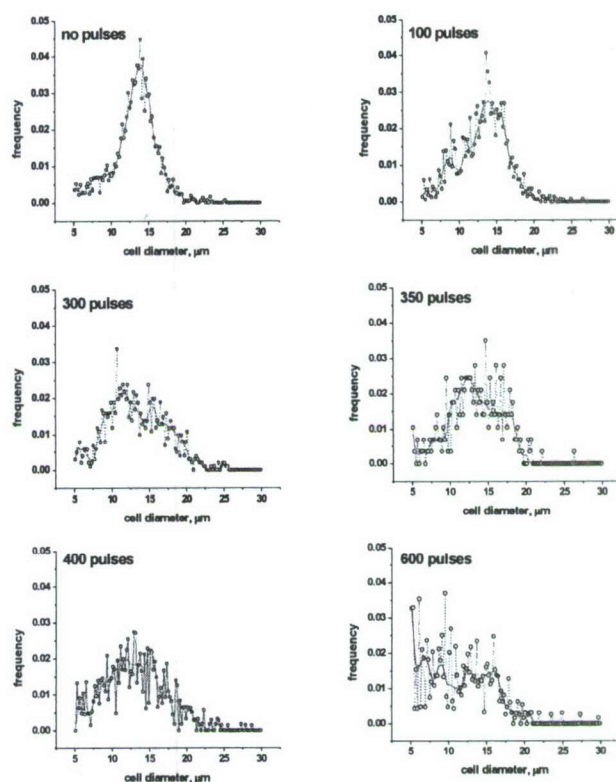


Figure 1. Change in cell diameter distribution following trains of 140kV/cm, 10 ns pulses. Except for 350 pulses, each point is the average of 2 or 3 replicates. The red lines are obtained by using a median smoothing function on the data.

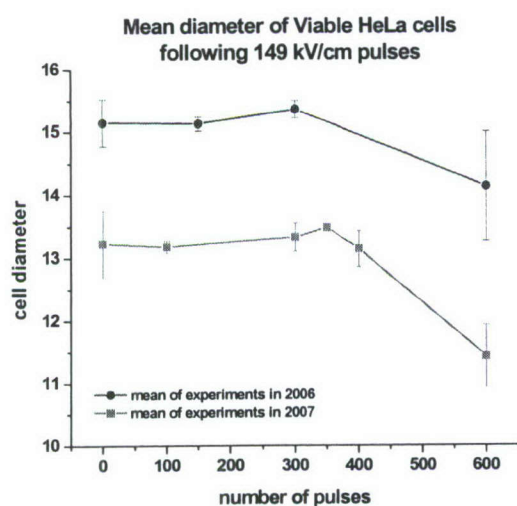


Figure 2. Although the mean diameter of the Hela cells used in these experiments are smaller than those from 2006, the mean diameters remained constant until 300-400 pulses were applied in both cases. After this threshold further pulses resulted in decreased cell diameters.

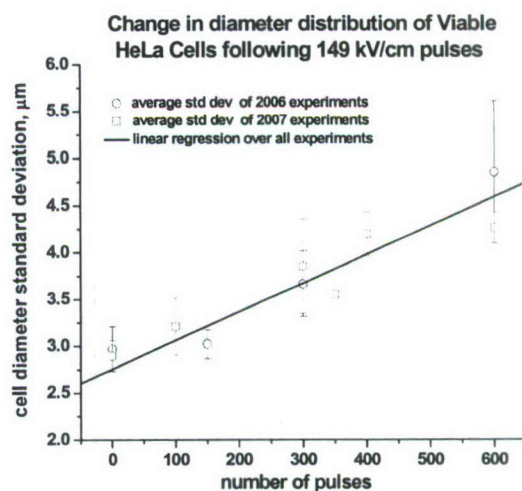


Figure 3. Change in cell size distribution following application of HE pulses. Although the distribution means are unchanged until the threshold has been exceeded, the spread of the diameter distributions increases at the lowest number of applied pulses.

poration, then the size of the pores are too small to allow trypan blue (MW 961) to cross. Furthermore, the clonogenic fraction of cells is unaffected below the threshold level (Figure 5).

Beyond the pulse threshold 3 observations are made: the mean of cell distribution decreases possibly indicating membrane disruption, the cell viability as measured by trypan blue exclusion decreases, and the clonogenic surviving fraction decreases. All three of these response variables continue to decrease with increased number of pulses. These data indicate that whatever damage occurs to the cells below the threshold level is repairable and does not result in cell death. However, once the threshold is exceeded the irreparable cell damage increases with increasing dose. A comparison to the clonogenic survival curve based on only the live cells plated to that based on the total cells plated indicates that much of the cell killing is immediate. This observation is consistent with our previous annexin V apoptosis data, which indicated apoptosis immediately after pulsing, but the annexin V positive cells were gone within a couple of hours after pulsing (those cells destined to die did so quickly).

The mitochondrial membrane potential was measured at 0, 200, 300, and 400 pulses (Figure 6). The upper left region is live cells with polarized membranes and the lower right region is dead or dying cells with depolarized membranes. An intermediate population appears when pulses are applied. This population increases with increased pulses. In a subsequent experiment it was shown that this intermediate population disappeared after 4 hours without a concurrent increase in depolarized cell population, indicating that most of cells had recovered.

A fractional factorial designed experiment was used to investigate the effects of electric field strength (80 and 140 kV/cm), pulse number (200 and 400), pulse frequency (1/sec and 0.5/sec), temperature (cells kept on ice except when pulsing and cells kept at room temperature) and agitation of the cells during pulsing to keep them from settling. The measured endpoints were cell viability and clonogenic surviving fraction. As expected from previous result, the electric field strength and pulse number had strong effects and the non-additive interaction between the two was significant. The cells kept on ice were slightly more resistant to the HE pulses than cells kept at room temperature. And although it seems counterintuitive, there was less cell killing with the higher number of pulses when they were given at a higher frequency. A manuscript summarizing this work is being prepared for publication.

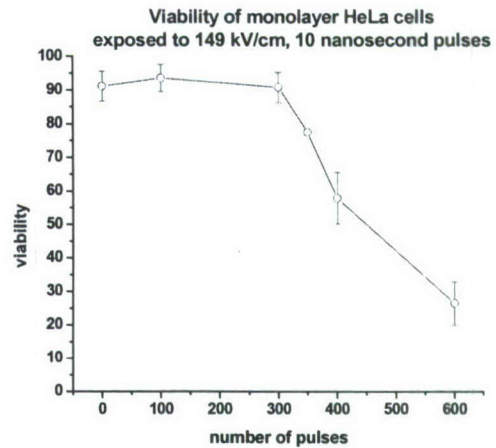


Figure 4. Viability of cells after pulsing (determined by trypan blue exclusion). There is no cell killing until after 300 pulses are applied.

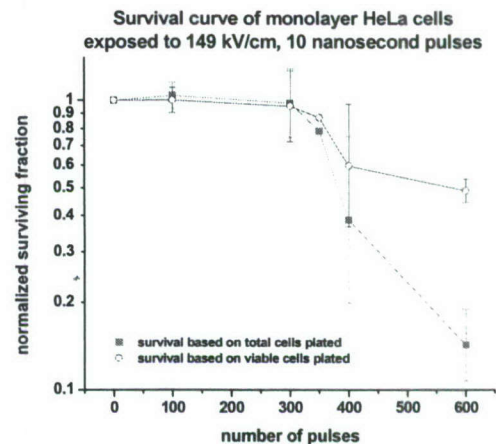


Figure 5. Clonogenic survival after pulsing. There is no cell killing until after 300 pulses are applied. The difference between the curve based on live cells and total cells plated indicates that much of the cell killing is immediate.

## Bioreactor for investigating the efficacy of HE pulses on 3 dimensional cell aggregates under controlled tumor microenvironments.

HeLa cells grown as 3-D aggregates (spheroids) were shown using clonogenic survival curves and viability (trypan blue exclusion) to be more resistant to the HE pulses than individual cells exposed in suspension. An attempt was made to convert an electroporation cuvette into a bioreactor that would be compatible with the existing bioreactor control system in our lab. In a bioreactor, spheroids, which better mimic tumors *in vivo*, could be exposed to HE pulses under conditions that mimic the tumor microenvironment (low O<sub>2</sub>, low pH, low glucose, etc). The pulse trace on the cuvette filled with glass beads, which are needed as a matrix to entrap the spheroids, was similar to that obtained without the beads, but the measured electric field for a given pSF<sub>6</sub> was higher. Because the media flow rate had to be very low due to the 2mm restriction of the cuvette, sufficient O<sub>2</sub> and CO<sub>2</sub> to maintain cell viability and pH control could not be transferred into the system. Designs to overcome this limitation are conceivable, but they would not be compatible with the existing control system.

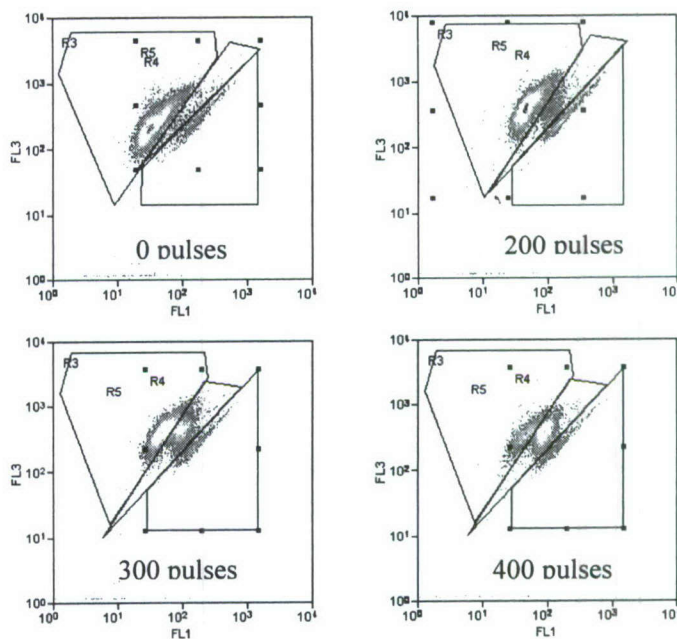


Figure 6. Mitochondrial membrane potential following application of 150 kV/cm 10 ns pulses. The upper left region is live cells and the lower right region is dead or dying cells. An intermediate population appears with the application of pulses and increases with pulse number.

**Effect of Broadband Radiofrequency Microwaves on Gene Expression.** This work and results have been published (see below)

## II. Personnel, Publications, Interactions, Patents

### A. Personnel Supported

**Joseph L. Roti Roti, Washington University School of Medicine, Principal Investigator**

Timothy D. Whitehead, Staff Scientist,

Ryuji Higashikubo, Research Associate Professor

### B. Refereed Publications (2007)

Whitehead, T.D., E.G. Moros, B.H. Brownstein, and J.L. Roti Roti, *Gene expression does not change significantly in C3H 10T(1/2) cells after exposure to 847.74 CDMA or 835.62 FDMA radiofrequency radiation.* Radiat Res, 2006. **165**(6): p. 626-35.

Whitehead, T.D., E.G. Moros, B.H. Brownstein, and J.L. Roti Roti, *The number of genes changing expression after chronic exposure to Code Division Multiple Access or Frequency DMA*

*radiofrequency radiation does not exceed the false-positive rate.* Proteomics, 2006. 6(17): p.4739-44.

Whitehead, T.D., B.H. Brownstein, J.J. Parry, D. Thompson, B.A. Cha, E.G. Moros, B.E. Rogers, and J.L. Roti Roti, *Expression of the proto-oncogene Fos after exposure to radiofrequency radiation relevant to wireless communications.* Radiat Res, 2005. 164(4 Pt 1): p. 420-30.

### **C. Interactions**

This data was shared with Dr. Ravi Joshi for use in his model development.

## University of Wisconsin

### I. Achievements

UW-Madison, in collaboration with other MURI consortium investigators accomplished a number of important research advancements regarding the interactions of pulsed electric fields (PEFs) with biological cells and cell-specimens. A brief summary of our research contributions is provided below. In addition, MURI funding supported the completion of one Ph.D. dissertation and one M.S. thesis, laid the foundation for a second Ph.D. dissertation, and supported the research of 6 undergraduate independent study students. To disseminate the research findings of these students, two journal articles have been published, one article is successfully undergoing a second review, and two more are in preparation. The MURI grant funded the development of a new research facility for real-time, in-situ fluorescence microscopy studies of PEF effects on biological cells, along with a new method to fabricate precisely-dimensioned, mechanically robust microcuvettes for use in these PEF-induced fluorescence experiments.

In collaboration with Dr. M. Meltz, detailed electromagnetic simulations demonstrated that TEM chambers are not an ideal configuration for delivering controlled pulsed electric field exposures to biological cells. The typical configuration involves placing a specimen flask containing a suspension of cells in culture medium in the middle of the TEM chamber. In the absence of the specimens, spatially uniform, monopolar, nanosecond pulses of intense electric (electromagnetic) fields can be generated inside these TEM chambers. However, once the chambers are loaded with the biological specimens, the field distributions and temporal waveforms *inside* the flask medium (i.e., experienced by the biological cells) are very complicated and generally not particularly intense, due to poor electromagnetic coupling and dielectric resonator reverberation effects. In the absence of the specimens, the pulses can be engineered to be monopolar, which is known to be ideal for stimulating membrane responses such as electroporation. In the presence of the specimen, the fields experienced by the cells are generally bipolar, with multiple oscillations of the electric field polarity, which is known to tend to suppress membrane electroporation response. Use of impedance-matching substrates and other TEM chamber "furniture" can improve the situation, but effective designs are generally not intuitive and require 3D electromagnetic field simulations. On the positive side, these 3D FDTD electromagnetic field simulations *were* able to provide highly precise thermal dosimetry and establish that any effects observed by cells exposed to *ns* pulses in TEM cells are due to nonthermal electric field influences. This work was published in ["FDTD Analysis of a Gigahertz TEM Cell for Ultrawideband Pulse Exposure Studies of Biological Specimens," Z. Ji, S.C. Hagness, J.H. Booske, S. Mathur, M. Meltz, *IEEE Trans. Biomed. Engr.* Vol. 53, no. 5, 780-789 (2006)].

Out of collaborative discussions with ODU and EVMS colleagues (Kolb, Schoenbach, Beebe, Beuscher), a new experimental configuration was designed and constructed at UW-Madison. This facility enabled fluorescence microscopy studies of the real-time, prompt response of biological cells after intense PEF exposure. By being able to generate PEFs with continuously-variable pulselengths between 60 to  $\infty$  ns, it provided capabilities that complemented those developed at ODU for ns and sub-ns PEFs. In particular, it provided a means to study cellular responses spanning the divide between the ultra-short ns regimes and the 100's of microseconds to millisecond exposures common with commercial electroporation instruments. This system is described in [Z. Ji, S.M. Kennedy, J.H. Booske, and S.C. Hagness, *IEEE Trans. Plasma Sci.* Vol. 34, no. 4, 1416-1424 (2006)].

We developed a new method to batch-microfabricate micro-cuvettes (on optical microscope slides) for use in fluorescence-microscopy-analyzed electroporation experiments. We were successful at fabricating these microcuvettes on conventional optical microscope slides (for conventional fluorescence microscopy) as well as glass cover slips (for confocal microscopy, requiring a thinner glass substrate). As a result of the popularity and performance capabilities of these slides, we manufactured and delivered batches to collaborators at ODU, University of Michigan, and Loughborough University (UK). In our

own experiments at UW-Madison, we exploited their mechanical robustness to conduct hundreds of repeat experiments with a single microcuvette. The ability to easily but precisely fabricate an exposure gap of less than 100  $\mu\text{m}$  has enabled us to repeatedly and reliably achieve high electric field exposure intensities (up to  $10^7$  V/m) with relatively modest voltages ( $< 1.5$  kV). Furthermore, using finite element electromagnetic modeling, validated by careful experimentation, we have been able to obtain detailed knowledge of the exact electric field distributions experienced by cells contained in the microcuvette. This has been possible because of our ability to precisely manufacture and measure the dimensions of the microcuvette's micro-gap. These microcuvettes are described in [Stephen M. Kennedy, Zhen Ji, Jonathan C. Hedstrom, John H. Booske, Susan C. Hagness, "Real Time Quantification of Electroporative Uptake and Field Heterogeneity Effects in Cells," *Biophysical Journal*, (in review, 2008)].

By measuring the uptake of Trypan Blue after PEF exposures, we were able to experimentally study the dynamics behind pore formation and recovery, including the effect of single pulses of variable pulsewidth, pulse trains of fixed interpulse spacing, pulse trains with different interpulse spacing, and the effect of calcium and magnesium extracellular concentrations. Estimates of the required pulse ohmic dissipation energy delivered to cell populations were analyzed resulting in a pulse energy curve exhibiting a local minimum, in agreement with theoretical predictions made by Joshi and Schoenbach [R. P. Joshi and K. H. Schoenbach, *Phys. Rev. E*, vol. 62, no. 1, pp. 1025–1033, 2000]. Our research identified that the local minima characteristics of this pulse energy curve can be usefully related to both the pore relaxation time and the pore-diffusion-time constant, a fundamental parameter associated with the rapidity with which pore size can change in response to a driving force. A quantitative estimate for the product of the pore diffusivity and the line-tension pore energy parameter can be inferred from our data. A comparison with the values assumed in previous simulation studies suggest that the diffusivity and/or the line-tension parameters are smaller than previously expected, at least for HL-60 cells. Meanwhile, variable interpulse spacing was employed to examine different attributes of membrane dynamics (pore generation, evolution, size distribution, and resealing time). These measurements indicated a transient pore relaxation or resealing time of approximately 250 ns for HL-60 cells, in close agreement with the value inferred from the pulse-energy-versus-pulsewidth data. For pulse trains, the required electric field decreased monotonically with the number of pulses. This was explained in terms of a pulse accumulation effect. Finally, the effect of increasing the cell suspension medium's  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  ionic concentration was observed to impede electroporation. These results are all reported in [Z. Ji, S.M. Kennedy, J.H. Booske, and S.C. Hagness, *IEEE Trans. Plasma Sci.* Vol. 34, no. 4, 1416-1424 (2006)].

We have conducted experiments quantitatively investigating electroporative uptake kinetics of a fluorescent plasma membrane integrity indicator, propidium iodide (PI), in HL60 human leukemia cells resulting from exposure to 40  $\mu\text{s}$  pulsed electric fields (PEFs). These experiments were made possible by our precision microcuvettes and by our successful development of an *in situ* real-time fluorescence intensity calibration within these microcuvettes. Our finite-element electrostatic simulations quantifying the electric field heterogeneity between the microcuvette's electrodes allowed us to correlate trends in electroporative response to electric field distribution. Analysis of experimental data identified two distinctive electroporative uptake signatures: one characterized by low-level, decelerating uptake beginning immediately after PEF exposure and possibly persisting for 100s of seconds. The other response involves high-level, accelerating fluorescence that is manifested sometimes hundreds of seconds after PEF exposure. The qualitative nature of these fluorescence signatures were used to isolate the conditions required to induce exclusively transient electroporation and to discuss electropore stability and persistence. For HL60 cells exposed to 40  $\mu\text{s}$  PEFs, a range of electric field strengths resulting in transient electroporation was identified existing between 1.6 and 2 kV/cm. Quantitative analysis was used to determine that HL60s experiencing transient electroporation internalized between 50 and 125 million nucleic acid-bound PI molecules per cell. Finally, we demonstrated that electric field heterogeneity may be used to elicit asymmetric electroporative PI uptake within cell cultures and within

individual cells. These results are reported in [Stephen M. Kennedy, Zhen Ji, Jonathan C. Hedstrom, John H. Booske, Susan C. Hagness, *Biophysical Journal*, (in review, 2008)].

The long time intervals after PEF exposures during which PI uptake was observed to persist is surprising (500-600 s). Previous model predictions do not explain how electropores would remain open and stable for hundreds of seconds. We have developed a modified electropore dynamics model based on the Smoluchowski equation that provides possible explanations for the observance of long-lived electroporatively-induced molecular uptake. This model considers finite pore length, voltage-dependent electropore current density and locally constrained membrane surface tension based on cytoskeletal anchoring points about the plasma membrane. Analysis of simulation data reveals that a number of large electropores may remain open for a significant period of time after the cessation of the applied PEF. We are now examining how well this model predicts experimentally observed amounts of PI uptake when using 1  $\mu$ s PEFs. These results are reported in [Z. Ji, *Ph.D. dissertation*, UW-Madison (2007)].

We are continuing our research after the MURI, by emphasizing the prospects for synergistically combining spatiotemporal heterogenous control of electrical and chemical environments to improve molecular uptake efficiency in cells.

## **II. Personnel, Publications, Interactions, Patents**

### **A. Personnel Supported**

#### **John H. Booske, Principal Investigator**

Susan C. Hagness, Co-Principal Investigator

Zhen Ji, Graduate research assistant (Electrical Engineering)

Steve Kennedy, Graduate research assistant and previous undergraduate student

Poliang Lin, Undergraduate student

Lorena Santos, Undergraduate student

Mike Hitchcock, Undergraduate student

Jonathan Hedstrom, Undergraduate student

Rafael Vilorio, Undergraduate student

Nicole Rockweiller, Undergraduate student

### **B. Publications and Presentations**

#### **PUBLICATIONS IN REFEREED JOURNALS**

"FDTD Analysis of a Gigahertz TEM Cell for Ultrawideband Pulse Exposure Studies of Biological Specimens," Z. Ji, S.C. Hagness, J.H. Booske, S. Mathur, M. Meltz, *IEEE Trans. Biomed. Engr.* Vol. 53, no. 5, 780-789 (2006).

"Experimental studies of dynamic cellular membrane response by electroporation of HL- 60 cells," Z. Ji, S.M. Kennedy, J.H. Booske, and S.C. Hagness, *IEEE Trans. Plasma Sci. (Special Issue on Non-Thermal Medical/Biological Applications of Ionized Gases and Electromagnetic Fields)* Vol. 34, no. 4, 1416-1424 (2006).

Stephen M. Kennedy, Zhen Ji, Jonathan C. Hedstrom, John H. Booske, Susan C. Hagness, "Quantification of Electroporative Uptake Kinetics and Field Heterogeneity Effects in Cells," *Biophysical Journal*, (in review, 2008).

"Numerical analysis of pulsed electric field induced cell membrane dynamics," Z. Ji, S.M. Kennedy, N.B. Rockweiler, J.H. Booske, and S.C. Hagness, in preparation (2008).

"Persistent cell membrane poration and dye uptake," Z. Ji, S.M. Kennedy, J.H. Booske, and S.C. Hagness, in preparation (2008).

## **PUBLISHED ABSTRACTS**

"Development and characterization of the microcuvetted: an exposure device for real-time observation of electroporative molecular uptake," S.M. Kennedy, Z. Ji, J.H. Booske, and S.C. Hagness, Bioelectromagnetics Society Annual Meeting, San Diego, CA, June 8-12 (2008).

"Real-time quantification of electroporative uptake kinetics and electric field heterogeneity effects in cells," S.M. Kennedy, Z. Ji, J. Hedstrom, J.H. Booske, S.C. Hagness, Bioelectromagnetics Society Annual Meeting, San Diego, CA, June 8-12 (2008).

"Quantification of Electric-Field-Induced Molecular Uptake Kinetics in Human Leukemia Cells and the Regulatory Influence of Electric Field Heterogeneity," Stephen M. Kennedy, Zhen Ji, Jonathan C. Hedstrom, John H. Booske, and Susan C. Hagness, XXIX General Assembly of the International Union of Radio Science (URSI), Chicago, IL, Aug 7-13 (2008).

"Experimental and numerical studies of molecular uptake dynamics in HL-60 cells induced by pulsed electric fields," Stephen M. Kennedy, Zhen Ji, John H. Booske, Susan C. Hagness, James C. Weaver, IEEE Pulse Power and Plasma Science Conference, June 17-22, Albuquerque, NM (2007).

"Studies of Persistent Poration Dynamics of Cell Membranes Induced by Electric Pulses," Z. Ji, S.M. Kennedy, J.H. Booske, S.C. Hagness, Bioelectromagnetics Society, Cancun, Mexico, 11-15 June (2006).

"Studies of Persistent Poration Dynamics of Cell Membranes Induced by Electric Pulses," Z. Ji, S.M. Kennedy, J. Booske, and S. Hagness, International Conference on Plasma Science, 4-8 June (Traverse City, MI, 2006).

"Experimental studies of relaxation time constants and membrane memory effects by quantitative measurements of PI-uptake of HL60 cells using sub-microsecond multi-pulse electroporation," Z. Ji, S. M. Kennedy, J. H. Booske, and S. C. Hagness, ElectroMed 2005, Portland, OR 15-18, May, 2005.

"Finite Difference Time-Domain (FDTD) Analysis and Dosimetry of a Gigahertz TEM Cell," Z. Ji, S.C. Hagness, J.H. Booske, S. Mathur, and M. Meltz, 26th Annual Meeting of the Bioelectromagnetics Society, June 20-24, Washington, DC (2004).

"Studies of Biological Specimens," Z. Ji, S.C. Hagness, J.H. Booske, S. Mathur, and M. Meltz, IEEE AP-S International Symposium on Antennas and Propagation and USNC/URSI National Radio Science Meeting, Monterey, CA, June 20-26 (2004).

"Subcellular Response to Pulsed Narrowband RF Radiation," Zhen Ji, Susan C. Hagness, John H. Booske, Symposium Record Abstracts, ElectroMed 2003, San Antonio, TX, June 11-13, 2003.

### **C. Interactions and Activities Related to the MURI Project**

Interactions with Purdue MURI consortium scientists at Purdue Calumet, Summer-Fall 2002, measurements of microwave dielectric properties of cell culture media with different concentrations of cells. UW provided microwave measurement instrumentation and expertise.

Collaboration with scientists at University of Texas Health Science Center (M. Meltz) and Brooks City Air Force Base (S. Mathur), investigating ultrawideband (UWB) radiation pulse dosimetry during laboratory exposure of cell-containing culture medium specimens in GTEM exposure chambers. UW provided 3D FDTD computational capabilities.

Close consultations and interactions with ODU and EVMS colleagues (Schoenbach, Jurgen, Buescher, Beebe) while setting up fluorescence microscopy and High Voltage pulse exposure system at UW.

Consultations and collaborations with K. Schoenbach (ODU) and J. Weaver (MIT) in identifying UWB pulse exposure research directions that would closely interface with consortium modeling of cells.

Collaborative discussions with J. Weaver (MIT), R. Joshi (ODU), and S. Beebe (EVMS), both in preparation for, and during the ElectroMED 2005 Conference, on the design of electroporation experiments at UW to test prevailing theoretical hypotheses. An article on this work was published in 2006.

Developed a protocol to batch-microfabricate microcuvettes (on optical microscope slides) for use in fluorescence-microscopy-analyzed electroporation experiments. Initiated a collaboration with K. Schoenbach, J. Kolb, and colleagues at ODU, Professor Michael Kong of Loughborough University (UK), and Professor Ron Gilgenbach at the University of Michigan to evaluate and use these new microcuvettes in Consortium and other experiments. Microcuvettes have been produced and distributed to collaborating groups and were extremely well received. Extra thin microcuvettes (on microscope cover slips) were produced and given to K. Schoenbach, J. Kolb, and colleagues at ODU for use in experiments requiring very close objective lens placement. Details on microcuvette fabrication were provided to J. Weaver (MIT). A publication providing details of microcuvette fabrication and its use to observe quantitative, real-time molecular uptake is being reviewed for publication.

Collaborative discussions with R. Joshi (ODU), J. Weaver (MIT) and J. Kolb (ODU) are ongoing in planning simulation studies and further experiments to understand the mechanisms responsible for observations by both our students and by personnel at ODU on delayed and prolonged uptake of fluorescence molecules in cells exposed to microsecond and submicrosecond electroporation pulses. Two manuscripts are being prepared on this simulation work.

Professor William Murphy (Biomedical Engineering, UW) has also been brought into discussions as a collaborator. We are working with him to develop electro-stimulatory techniques to obtain spatial and temporal control over genetic expression within 3-dimensional, biologically inert lattices for use in regenerating functional tissues from patient-derived stem cells.